

SYMBIOTIC NITROGEN FIXATION IN TWO SPECIES
OF LEGUMINOSAE: LATHYRUS MARITIMUS (L) BIGEL.
AND OXYTROPIS CAMPESTRIS (L) DC.

CENTRE FOR NEWFOUNDLAND STUDIES

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JOHN BARIMAH-ASARE, B.Sc.(HONS.)



SYMBIOTIC NITROGEN FIXATION IN TWO SPECIES OF LEGUMINOSAE:
LATHYRUS MARITIMUS (L) BIGEL. AND *OXYTROPIS CAMPESTRIS* (L) DC.

BY

© JOHN BARIMAH-ASARE, B.Sc. (HONS.)

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ABSTRACT

The symbiotic association of *Rhizobium* strains and two species of legumes, *Lathyrus maritimus* (L.) Bigel. and *Oxytropis campestris* (L.) DC found in subarctic regions of Newfoundland were investigated. The root nodule bacteria were isolated and routinely characterized using standard techniques for *Rhizobium* species. The isolate of *L. maritimus* was found to resemble fast-growing *Rhizobium leguminosarum* biovar *vicia*, except in its ability to tolerate a wide range of pH and a high concentration of NaCl. The isolate of *O. campestris* exhibited characteristics which resembled those of both slow-growing and fast-growing strains. Both isolates could grow, although slowly, at a low temperature (5°C).

Cross inoculation tests were performed using the isolates on other legume species. The isolate of *O. campestris* could not infect any of the hosts used while that of *L. maritimus* could infect *Vicia cracca*.

Growth patterns and external morphology of the symbiotic root nodules were studied. The beaded structure of field-collected nodules indicate that they resume growth after overwintering annually. The results of laboratory-grown specimens showed that nodules of both species attain a maximum size after which no growth occurs. Nitrogen fixation was assessed in the legumes using the acetylene reduction method with nodulated root systems. The two species showed highest nitrogenase activity at 20°C and were still capable of

activity at low temperatures; for example, nodules of *L. maritimus* and *O. campestris* were able to maintain 35% and 80% of their maximum activities respectively at 5°C, showing their adaptation to nitrogen fixation in the cold.

The anatomy of *L. maritimus* root nodules was studied using light and electron microscopy. They were found to possess a zonal differentiation from the tip to the point of attachment to the root; i.e. the meristem, invasion zone, early symbiotic zone, late symbiotic zone and the senescent region. Nodules possessed lipid bodies which were more abundant in the cortical cells. The lipid bodies may be involved in the protection of the nitrogen-fixing tissue from the cold environment by forming an insulating jacket around it.

These studies indicate that the legumes and their symbionts are adapted to function effectively at low temperature and therefore could be of significance in land reclamation and for other purposes in marginal northern agricultural areas. The unique characteristics shown by the isolates can be useful to other *Rhizobium* and *Bradyrhizobium* strains by transferring them, using DNA recombinant techniques.

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I. INTRODUCTION

I.1. General introduction:

Nitrogen is one of the most abundant components of living organisms. As a constituent of essential bio-molecules such as proteins and nucleic acids it serves an important functional and structural role in all living systems. It is found mainly as an inert dinitrogen gas in the atmosphere and cannot be assimilated by living organisms. Living organisms derive their nitrogen in a combined form from an inorganic source by uptake and assimilation of nitrogenous compounds, through ingesting other life forms or through biological nitrogen fixation.

Biological nitrogen fixation is a process carried out by certain prokaryotic cells (Gallon and Chaplin 1987), collectively called diazotrophs, which lead to the conversion of dinitrogen gas in the atmosphere to a form that can be assimilated by living organisms. Other phenomena, such as lightning and ultraviolet radiation, provide nitrogen to living systems, to a lesser extent, by combining nitrogen and oxygen gases in the atmosphere and subsequently transforming them into nitrates and nitrites which can also be assimilated by living organisms. Table 1 gives a list of some methods of nitrogen-fixation and their relative contribution to the world nitrogen budget.

Some of the useful, combined nitrogen is lost through leaching, surface run-off and the activities of denitrifying bacteria whose action results in the release of nitrogen gas. In natural ecosystems there is a cycling of nitrogen through plants, animals and microbes which is maintained in a proper balance. An increasing human population and a higher demand for food have led to clearing of natural ecosystems for intensive agricultural practices. The result is a disturbance of the balance in the nitrogen budget. Nitrogen therefore has to be continually supplied in order to maintain or increase productivity. It can be applied as nitrogenous chemical fertilizers or through biological means; which one of the two is a better alternative has been a contentious issue (Subba Rao 1980). Although both are energy-demanding, the cost of the industrial production of chemical fertilizers has been rising significantly in recent times thereby favouring biological nitrogen-fixation (Subba Rao 1988). Biological nitrogen-fixation, requiring a relatively simple and localized technology using largely renewable resources, will play an increasingly important role in world agriculture in the future.

Biological nitrogen-fixation has been estimated to contribute about 175×10^6 metric tonnes of nitrogen to the earth's ecosystem annually, with nodulated legumes grown for agricultural purposes accounting for about one quarter of that value (Burns and Hardy 1975).

Table 1. Different modes of nitrogen-fixation and the relative contribution of each to the worldwide nitrogen economy:

Method	Rate*	Reference
Biological fixation	101-175	Subba Rao, 1980
Industrial fixation	40	Subba Rao, 1980
Lightning	10	Burns and Hardy, 1975

(*) million metric tons per annum

The rest is fixed by other biological systems like free-living bacteria and non-cultivated legumes. For instance, non-cultivated legumes have been found to contribute significantly to the nitrogen budget of the tundra soils of the Northwest Territories, Canada (Karargatzides et al. 1985) and Alaska (Alexander et al. 1978). Also, some cyanobacteria fix nitrogen in the free-living form while others do so only when they form symbiotic relationships with other organisms like algae, fungi, bryophytes, pteridophytes and higher plants. Table 2 shows estimates of the amount of nitrogen fixed by some biological systems.

Prokaryotic organisms (examples given in Table 3) fix atmospheric nitrogen in a reaction catalysed by the enzyme nitrogenase. This reaction can be expressed stoichiometrically as:



The reduction process is energetically expensive because electrons of low redox potential and energy in the form of ATP have to be made available to the endergonic reaction (Yates 1980).

Table 2. Estimates of annual biological nitrogen-fixation on earth (Data extracted from Burris, 1977).

Systems	Rates*
Legumes	35
Non-legumes	9
Permanent grassland	45
Forest and woodland	40
Unused land	10
Ocean	36

(*) million metric tons per annum

Table 3. Some biological nitrogen-fixing prokaryotes (Modified from Gallon and Chaplin, 1987; and Postgate, 1980):

Category	Genus	Species
Asymbiotic diazotrophs		
Strict anaerobes	<i>Clostridium</i>	<i>C. pasteurianum</i>
		<i>C. butyricum</i>
Facultative anaerobes or microaerobes	<i>Enterobacter</i>	<i>E. cloacae</i>
		<i>E. aerogenes</i>
	<i>Klebsiella</i>	<i>K. pneumoniae</i>
	<i>Citrobacter</i>	<i>C. freundii</i>
Obligate aerobes	<i>Azospirillum</i>	<i>A. lipoferum</i>
	<i>Azotobacter</i>	<i>A. vinelandii</i>
	<i>Beijerinckia</i>	<i>B. derxii</i>
Photosynthetic bacteria	<i>Rhodospirillum</i>	<i>R. rubrum</i>
	<i>Rhodopseudo-</i> <i>monas</i>	<i>Rp. capsula</i>
Chemoautotrophs	<i>Thiobacillus</i>	<i>T. ferrooxidans</i>
	<i>Methylosinus</i>	<i>M. trichosporum</i>
	<i>Xanthobacter</i>	<i>X. autotrophicus</i>
Symbiotic diazotrophs	<i>Rhizobium</i>	<i>R. leguminosarum</i>
		<i>R. meliloti</i> , <i>R. loti</i>
	<i>Bradyrhizobium</i>	<i>B. japonicum</i>
	<i>Frankia</i> sp.	

Symbiotic associations of legumes and *Rhizobium* or *Bradyrhizobium* species lead to the formation of root nodules which are the sites of nitrogen-fixation. In these systems the host plants supply photosynthates which are oxidized to provide the energy requirement. These associations have attracted considerable attention because they are very important in food and fibre production (Evans and Berber 1977). In spite of the obvious importance to agriculture in particular and their interest to biology in general only a small number of legumes have been studied extensively. Allen and Allen (1981) reported that only 16% of legume species have been examined for nitrogen-fixing root nodules. A still lesser proportion have had their nodules studied in detail. Moreover, most of the knowledge of the symbiotic association and the bacterial species involved is restricted to cultivated legumes. Information about the biology of non-cultivated legumes is needed as they may have some special properties. Also the symbionts may be manipulated, because of their special characteristics, through DNA recombinant techniques to produce more effective strains for nitrogen-fixation.

1.3. *Rhizobium* taxonomy:

The taxonomy of *Rhizobium* has recently undergone numerous revisions with the division of the genus *Rhizobium* into two genera, the fast-growing genus *Rhizobium* and the slow-growing genus *Bradyrhizobium*. Together with the genus *Agrobacterium* they form the family Rhizobiaceae which has members that excite cortical hypertrophies on plants (Jordan 1984).

The names, *Rhizobium* and *Bradyrhizobium*, were derived from the Greek nouns *rhiza* meaning "root" and *bios* meaning "life"; hence *Rhizobium* "that which lives in the root" and the Greek adjective *bradus* meaning "slow" that is slow-growing *Rhizobium* (Jordan 1984).

Classification of the genera, *Rhizobium* and *Bradyrhizobium*, relies on a character which has a disputed taxonomic value; that is their ability to nodulate certain legumes and non-legumes like *Parasponia* species. The fact that the ability to nodulate a specific host could be lost or reduced if the bacterial strains are cultured for extended periods (Vincent 1970) means host nodulation ability is not a stable taxonomic character and therefore inappropriate for classification. Jordan and Allen (1974) have stated that the taxonomic position of *Rhizobium* is controversial and that the current classification can be regarded, at best, as tentative.

Consequently, the taxonomy of *Rhizobium* is in constant dispute and is continuously undergoing changes (Sprent 1981).

At the species level Fred et al. (1932) proposed the concept of "cross inoculation group" as the basis for *Rhizobium* taxonomy. They defined a "group" as consisting of plant species amongst which rhizobia are freely interchangeable in terms of ability to excite nodule formation. This concept led to the classification of *Rhizobium* into the major groupings which are still in use; i.e., *R. meliloti*, *R. leguminosarum*, *R. loti*, *R. trifoli* and *B. japonicum* (Jordan 1984). This classification is now known to be seriously flawed, especially with the tropical legumes (Bergesen 1982), because each of the species that results from this system of classification has now been shown to have members that are cross-infective with other groups to some degree (Graham 1976). Jordan (1984) also concludes that a classification based on the cross-inoculation or plant affinity concept is not satisfactory because of widespread anomalous cross-infections.

According to Jordan (1984) insufficient nodulation data is one of the limitations to effective *Rhizobium* taxonomy. He reported that of the 14,000 or so known species only 8 - 9% have been examined for nodulation and less than 0.5% have had their nodule bacteria studied and characterized.

1.3.1. Characterization of *Rhizobium* and *Bradyrhizobium*:

The most acceptable basis for assigning bacterial species or strains to the genus *Rhizobium* or genus *Bradyrhizobium* is their ability to form nodules on the roots of legumes or the non-legume, *Parasponia* species (Allen and Allen 1981). In addition, there are certain features with which every member of these two genera is expected to conform (Vincent 1970, Elkan 1984, Jordan 1984, Werner 1987). These are:

- a. the bacterial cells are Gram negative rods with some strains having a cocci structure in the initial phase of growth,
- b. the most preferred medium for growth is yeast extract mannitol (YEM) medium, producing watery or white colonies on solidified YEM,
- c. they are aerobic,
- d. the bacterial cells do not produce endospores,
- e. members of *Rhizobium* and *Bradyrhizobium* usually produce an acidic reaction and an alkaline reaction, respectively, on growth media,
- f. they utilize many carbohydrates and produce a considerable amount of extracellular slime during growth on a carbohydrate-containing media,
- g. colony size of 2-3 mm is produced by *Rhizobium* strains after 3 days and *Bradyrhizobium* strains after 7 days,
- h. the cells are motile.

1.3.2. Utilization of some carbohydrates and organic acids:

Some carbohydrates and organic acids are utilized as energy sources by *Rhizobium* and *Bradyrhizobium* strains (Graham 1964, Antoun et al. 1984). Some of the strains are able to utilize intermediate organic acids of the tricarboxylic acid (TCA) cycle as energy sources whilst others cannot. According to Trinick (1982), those strains that cannot metabolize these compounds possess a complete TCA cycle and therefore fail to actively transport them. Fast-growing *Rhizobium* and slow-growing *Bradyrhizobium* strains have been shown to display differences in the number and kind of carbohydrates that they can use (Vincent 1977). These differences have been used as criteria for characterizing root nodule bacteria. While *Rhizobium* can utilize a wide range of carbohydrates and organic acids as the only source of carbon for energy, *Bradyrhizobium* has a limited range (Graham 1964). Recently Stowers and Eaglesham (1984) used carbohydrate utilization as one of the criteria to differentiate between slow-growing and fast-growing strains of *Bradyrhizobium japonicum*.

The ability of a rhizobial or bradyrhizobial strain to grow on an agar medium with a particular carbohydrate or organic acid as the only source of carbon means it can utilize that carbohydrate or organic acid as a sole source of energy. Graham (1964) reported that carbohydrate utilization tests were clearly valid criteria for sub-divisions in the genus

Rhizobium. He further concluded that when used in conjunction with other diagnostic features, substrate utilization could permit a standard rhizobial identification.

I.3.3. Temperature effects:

The effect of temperature on rhizobial and bradyrhizobial growth and the mechanism involved is not very well understood. According to Jordan (1984) the temperature range for *Rhizobium* and *Bradyrhizobium* is highly strain-dependent but generally lies between 4-42.5°C. Optimum rhizobial growth occurs in the range of 20-28°C (Vincent 1977).

Rhizobia are tolerant of temperatures below 4°C but very little growth, if any at all, occurs at this temperature (Jordan 1984). According to Trinick (1982), symbionts of plant species growing in cooler areas survive low temperatures better than those of tropical or hotter regions. Prévost et al. (1987) have reported that *Rhizobium* strains isolated from some *Oxytropis* species collected from the Canadian arctic region could grow very well at 5°C.

I.3.4. Intrinsic antibiotic resistance:

Intrinsic resistance is the natural or inherent resistance of a bacterial strain to certain antimicrobial agents. Differences in intrinsic resistance have been used as

a basis for bacterial identification, classification and for other taxonomic purposes (Cowan 1974).

Resistance to low concentrations of antibiotics has been found to be a stable property of bacteria and therefore a useful means of characterization of *Rhizobium* and *Bradyrhizobium* strains (Josey et al. 1979). Intrinsic antibiotic resistance has been used for strain identification of *Rhizobium* and *Bradyrhizobium* strains (Pankurst 1977, Cole and Elkan 1979, Prévost et al. 1987). However, generalizations using this criterion cannot be made any further than the strain level; this is because *Rhizobium* and *Bradyrhizobium* strains are generally sensitive to a wide range of antibiotics and the strain - to - strain variation within a rhizobial grouping is large enough to make such generalization unrealistic (Trinick 1982).

I.4. Plant species

I.4.1 *Lathyrus maritimus* (L.) Bigel.:

The name "lathyrus" was derived from the Greek noun "lathyros" which means "a pea" or "pulse" There are about 130 species in the genus *Lathyrus* consisting of climbing and herbaceous perennials. This genus belongs to the tribe Viciae of the subfamily Papilionoideae and the family Leguminosae (Fabaceae).

There are both wild and cultivated species of *Lathyrus*. Native species occur along seashores, lake and stream banks, roadsides, waste lands and in thickets and meadows. Some members are used for soil cover and green manuring.

Lathyrus belongs to the so-called pea cross-inoculation group. The work of Carrol (1934), Conklin (1936), Bushnell and Sarles (1937) and Wilson (1939) confirmed the mutual relatedness of *Lathyrus*, *Pisum*, *Vicia* and *Lens* and their rhizobia.

According to Allen and Allen (1981), comparatively few members of the genus have been studied. The literature on the species *Lathyrus maritimus*, which is used in these studies, is very limited. Most of the work done on this genus has been concentrated on those species that cause lathyrism. Symptoms of lathyrism in man usually appear after eating seeds of the plant, commonly occurring as a paralysis of the muscles below the knee, pains in the back followed by weakness and stiffness of the legs and progressive locomotive incoordination.

I.4.2. *Oxytropis campestris* (L.) DC var. *johannensis* Fern.:

There are about 300 species of *Oxytropis* which occur throughout the north temperate, sub-arctic and arctic regions with some species being circumpolar in distribution (Polunin 1959, Böcher et al. 1968, Allen and Allen 1981). This genus belong to the tribe Galageae of the subfamily Papilionoideae

and the family *Leguminosae* (*Fabaceae*).

Nodulated specimens of *O. campestris* were first reported by Kharbush (1928) in the French Alps and many have been reported since (Allen and Allen 1981). Quite recently, some nodulated species of *Oxytropis* were sampled from the Canadian arctic region and their nitrogen-fixing capacity assessed by Schulman et al. (1988).

The preferred habitat of this species is sandy or gravely soil; growing near glacial lakes, dry plains or in meadows up to 4,000 meters altitude (Ingwersen 1958). It is a persistent plant because of its deeply penetrating root system and its ability to produce many seeds having long periods of viability.

I.5. Nodule morphogenesis and anatomy:

Nodules develop in the cortex of roots of legumes and the non-legume, *Parasponia*, after cells of compatible strains of *Rhizobium* and *Bradyrhizobium* attach to the root surface and are subsequently internalized in the cortical tissue. The initial step involved in this process is the recognition of the correct plant host by rhizobial or bradyrhizobial cells. Recognition is mediated by the induction of specific factors when the host and the symbiont come together. Rhizobia have been found to be chemotactic which may probably be due to specific plant attractants (Bergman et al. 1988, Caetano-

Anollés et al. 1988). Rhizobial and bradyrhizobial cells get attached to the root surface after recognition using their surface carbohydrates to bind with lectins produced on root surfaces by the compatible plant hosts (Gallon and Chaplin 1987). Lectins have been shown to determine specificity in the legume-*Rhizobium* interaction (Diaz et al. 1989).

After the initial attachment to the root hairs, the bacterial cells are transported to the root cortex. In most legume species the bacteria cells are carried by means of a tube-like structure called the infection thread which originates from the root cell wall enclosing the bacteria and grows towards the root cortex (Robertson and Lyttleton 1982). In some species, however, internalization occurs through cracks or wounds in the cell wall as described for *Arachis* (Chandler 1978) and *Stylosanthes* (Sprent 1989).

Once bacteria get internalized, cells of the inner cortical region are stimulated to divide, initiating the nodule meristem. This process is not well understood (Dart 1977, Newcomb 1981) but it is believed to involve chemical messages from the bacterial cells to the plant cells (Trunchet 1978). The meristem produces vascular and cortical cells to the outside and cells destined for the nitrogen-fixing zone to the inside. Bacteria are finally transformed into bacteroids which are capable of fixing atmospheric nitrogen into ammonia, in the infected nodule cells.

The spatial disposition adopted by the dividing cells of

the nodule meristem cause nodules of various legumes to differ in shape and gross anatomy (Bergersen 1982). Two main types of nodules have in effect been recognized (Sprent 1979):

a. Determinate nodules:

In this type, the meristematic cells are distributed in pockets throughout the whole nodule tissue. The bacterial cells are released into dividing plant cells; as a result, plant cells containing bacteroids are distributed throughout the central mass of the developing nodule. The meristematic cells finally cease to divide. Further increase in nodule size occurs only through cell enlargement. This type of development leads to nodules having a more or less spherical shape, example, nodules of *Glycine max* (soybean plant).

b. Indeterminate nodules:

This type possesses persistent meristems at their apices. Bacterial cells, carried by the infection threads, are released into cells of the nodule tissue below the meristematic region and are subsequently transformed into bacteroids. Because new cells are constantly being produced at the tip of the nodule, older cells are displaced towards the proximal end of its attachment to the root which ultimately senesce. This type of development leads to elongated

structures, with tissues of graded age from the growing point to the point of attachment to the root; i.e., there is a clear zonal differentiation from the apex to the base of the nodule. Examples: nodules of *Pisum*, *Medicago* and *Vicia*.

Another difference is that in determinate nodules the endodermis is closed and the cell walls are thickened to form a prominent scleroid layer while indeterminate nodules are open at the undifferentiated tip (Bergersen, 1982). There have been various reports on the histology of legume root nodules with the use of different terminologies to designate different tissues in the symbiotic organ (Dangeard 1926, Bieberdorf 1938, Allen and Allen 1954; Libbenga and Bogers 1974, Dart 1977, Newcomb 1981, Newcomb and Wood 1986 and Dixon and Wheeler 1986). For the indeterminate type, the nodule tissue generally consists of the meristematic region at the tip, the zone of cell infection and differentiation, the active nitrogen-fixing tissue and the senescing region found at the proximal end. However, this type of clearly designated zonation does not apply to determinate nodules because the meristematic tissue is distributed in pockets throughout the organ.

The bacteroid-containing cells are the most prominent cells in nodules and in some they comprise all the cells of the central tissue whereas in others they may be interspersed with smaller uninfected or interstitial cells. These uninfected cells have been reported to provide interfaces

where active metabolic exchanges take place (Sprent 1972, Newcomb and Tandon 1981). The infected cells have a central vacuole devoid of bacteroids. Nodules have amyloplasts or starch granules concentrated on the periphery of the infected cells near to the gas-filled intercellular spaces (Bergersen and Goodchild 1973). Lipid bodies have also been shown to be associated with nodules of some legume species (Newcomb and Wood 1986, Prévost and Bal 1989, Emerson and Bal 1988, Bal et al. 1989 and Jayaram and Bal 1991).

Pericycle cells, modified to form specialized transport cells with a wall labyrinth of greatly increased surface called the transfer cells (Pate and Gunning 1972, Gunning 1977) have been reported in nodules of some species (Pate et al., 1969). Newcomb and Peterson (1979) have presented an extensive report on the occurrence and ontogeny of transfer cells in nodules and lateral roots of representative species of the Leguminosae.

Although the shape and structure of effective root nodules varies amongst various legume species, they have significant features in common (Bergesen 1982). They all contain, centrally, a relatively large volume of parenchyma cells whose cytoplasm is packed with bacteroids. The infected cells of both types of nodule are relatively enlarged and tetraploid, and in some cases may even be of higher ploidy. The cortical cells in the nodules however remain diploid. The fact that infected cells are polyploid suggests that the extra

DNA must have some significance but this is not fully understood (Dixon and Wheeler 1986).

I.6. Factors that affect nodulation and nitrogen-fixation:

Effective nodulation of legumes by rhizobia and bradyrhizobia is known to depend on the persistence of the microorganisms in the soil. The persistence of microorganisms in the soil has been found to be greatly influenced by environmental factors such as soil pH, soil moisture, soil aeration, soil temperature and availability of nutrients (Pugashetti et al 1982, Vincent 1974). Some *Rhizobium* and *Bradyrhizobium* strains do persist and subsequently infect their hosts in soils where environmental conditions are harsh suggesting that these strains have successfully adapted to those conditions.

A well-established population of a *Rhizobium* or *Bradyrhizobium* strain in the soil is a prerequisite for the prompt establishment of the rhizosphere and nodulation of seedlings. Rhizosphere populations have been reported to be influenced by such factors as pH, soil-water content, types of cation present and the nature of organic nutrients exuded from the root (Bergesen 1982). He also reported that soil temperature affects rates of infection and nodule development as well as the longevity and rate of nitrogen-fixation of nodules.

Because root nodules are symbiotic organs, their functioning is affected by all the factors that affect either partner. Thus, environmental factors that affect plant vigour or lead to reduction in the rate of photosynthesis also result in low nitrogen-fixation. A decreased amount of light energy, lower air temperatures and increased wind speeds leading to increased rate of transpiration are some of the factors that may adversely affect plant growth and development (Gibson 1963). Growth and development of legumes are greatly influenced by nutrient availability. They also have increased requirement for molybdenum, sulphur, copper and cobalt (Evans and Russell 1971). Nitrogen-fixing legumes require more inorganic phosphate than plants growing with adequate soil nitrogen (Bergesen, 1982).

I.7. Nitrogen-fixation assay:

Many methods have been developed to directly or indirectly measure the rate of nitrogen-fixation in plants and other nitrogen-fixing systems (Bergersen 1980, Turner and Gibson 1980). These include the acetylene-ethylene reduction assay (Hardy et al. 1968), Kjeldahl analysis (Burris and Wilson 1957), ^{15}N -enrichment assayed by mass spectrometry (Burris and Wilson 1957), ^{15}N -incorporation assayed by radioactive counting (Nicholas et al. 1961), micro-Conway diffusion technique coupled with titrimetric (Mortenson 1961)

or calorimetric analysis of NH_3 (Dilworth et al. 1965), and N_2 - H_2 uptake (Mortenson 1964) or H_2 evolution (Bulen et al. 1965) assayed manometrically.

The acetylene-ethylene assay is most commonly used to estimate biological nitrogen-fixation rates, however the Kjeldahl analysis and isotopic analysis of samples exposed to $^{15}\text{N}_2$ have been used to some extent.

I.7.1. The acetylene-reduction assay:

The observation made by Schollhorn and Burris (1967) that nitrogen-fixation is inhibited by acetylene, and Dilworth (1966) reporting that the nitrogen-fixing enzyme, nitrogenase, is capable of converting acetylene to ethylene provided the basis for using the acetylene-ethylene method to measure nitrogen-fixation in living systems.

The chemical steps for the enzymatic reduction of nitrogen and acetylene are similar. They both require nitrogenase, an energy source and a reductant. The two reactions can be represented by the equations (Dilworth 1966):



The capacity of nitrogenase to convert nitrogen to ammonia is

measured by its capacity to reduce acetylene to ethylene.

The acetylene-ethylene reduction assay has been used successfully to estimate the amount of nitrogen fixed by nitrogenase preparations, bacterial cell cultures, detached nodules, nodulated root systems, whole plant systems and soil samples (Hardy et al. 1968, Gibson et al. 1976, Schulman et al. 1988). The advantages of this method are that the substrate used, i.e. acetylene, and the instrumentation are considerably cheaper than for the other methods. It is also simple, sensitive and quick (Dixon and Wheeler 1986, Hardy et al 1973).

I.2. Objectives.

The purpose of this study is:

1. to isolate and study the characteristics of *Rhizobium* or *Bradyrhizobium* strains associated with two legumes, *Lathyrus maritimus* and *Oxytropis campestris*, which occur in the subarctic regions of Newfoundland;
2. to investigate the nitrogen-fixing capacity of the root nodules of these legumes and make a comparative analysis between them and other legumes;
3. to study the structural characteristics of root nodules of these legumes;
4. to study the ability of the isolates to infect other legume species.

II. MATERIALS AND METHODS

II.1. Source of plant specimens and seeds.

The plant specimens and some of the seeds used in this investigation were collected in June and September, respectively, from locations in Newfoundland (Figure 1).

The plant specimens were removed from the soil with a trowel so that their roots and the soil immediately surrounding them were kept intact. They were placed in pots for transportation.

II.1.1. *Lathyrus maritimus* (L.) Bigel.

A perennial which develops from rootstocks, it has a rather dense, short pubescence with fleshy leaves and a prostrate habit (Figure 2). Specimens were collected from the coast in northern Newfoundland near Point Riche (50° 42' N; 57° 23' W). At the collection sites, the plants coexist with other species on the strand and may occasionally be sprayed with seawater. This species is distributed on coasts and shores from Greenland to Siberia and Japan (Fernald 1950). In North America it is mostly distributed in Labrador, Newfoundland and Quebec (Hitchcock 1952, Lamourex and Grandtner 1977, Scoggan 1950).

XI.1.2. *Oxytropis campestris* (L.) DC var. *johannensis* Fern.

A low, tufted perennial with a stout taproot and compound leaves (Figure 3). The leaflets are small and have hairs with a purplish green coloration. Specimens were collected from arctic-alpine habitats in northern Newfoundland near Cape Norman (51° 37' N; 55° 54' W). The collection sites were exposed, dry and windy with a gravelly soil. Plant growth in this area was sparse. This species occurs on calcareous rocks and gravels from James Bay to Maine (Fernald 1950).

II.1.3. Seeds.

Seeds of *L. maritimus* and *O. campestris* were collected from the same localities as the plant specimens. Seeds of *Vicia cracca* were collected at a roadside in St. John's, Newfoundland while seeds of *Vicia faba* and *Pisum sativum* were purchased from Gaze Seed Company, St. John's, Newfoundland.

Figure 1. Map of Newfoundland showing the locations where plant specimens and seeds were collected:

A (Cape Norman) - *O. campestris*

B (Point Riche) - *L. maritimus*

C (St. John's) - *V. cracca*

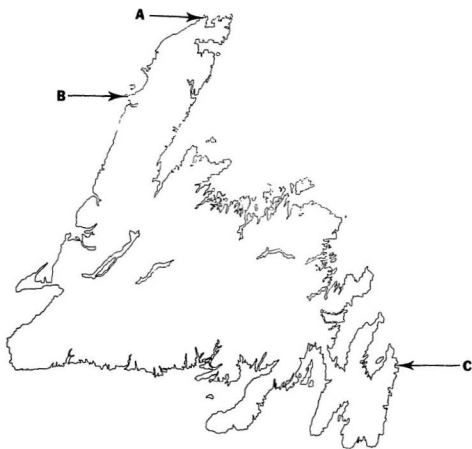


Figure 2. Photograph of *L. maritimus* taken at the collection site.



Figure 3. Photograph of *O. campestris* taken at the collection site.



II.2. *Rhizobium* and *Bradyrhizobium* strains.

II.2.1. Bacterial growth media preparation.

Yeast extract mannitol (YEM) medium is preferred by *Rhizobium* and *Bradyrhizobium* strains. The composition used was (Vincent 1970):

Mannitol	- 10.0 g
K_2HPO_4	- 0.5 g
$MgSO_4 \cdot 7H_2O$	- 0.2 g
NaCl	- 0.1 g
Yeast extract	- 0.5 g

made up to 1 litre with distilled water.

Solid medium was obtained by adding 1.5% weight by volume of agar (Difco Chemicals) to the YEM broth.

Amendments:

a: 1% volume by volume of 0.25% aqueous solution of congo red.

b: 0.5% volume by volume of 0.5% alcoholic solution of bromothymol blue.

c: 0.002% cycloheximide (Sigma Chemical Co.)

All bacterial growth media used were sterilized at 121°C for 15 minutes.

II.2.2. Sources, isolation and maintenance of bacterial cultures.

Root nodules were removed from the field-collected specimens and washed thoroughly with sterile distilled water. They were surface-sterilized by a momentary exposure to 95% ethanol, followed by washing with 3% hydrogen peroxide for 3-6 minutes depending on the size of the nodule (Vincent 1970). The nodules were crushed aseptically in sterile distilled water and the resulting suspensions streaked on YEM agar plates (amended with 0.002% cycloheximide to suppress fungal contamination), and incubated at 25°C for 5-10 days. Single colonies were selected and restreaked on agar plates amended with congo red to check for contaminants. Contaminants take up the stain and are coloured bright red while rhizobial colonies do not and are colourless or pinkish (Vincent 1970). After 7 days single colonies were streaked on YEM agar slants, incubated at 25°C for 7 days and stored at 4°C.

Rhizobium leguminosarum biovar *vicia* was obtained on agar slants from the collection of the Department of Biology, Memorial University of Newfoundland and maintained at 4°C.

Bradyrhizobium sp 32HI was obtained from Nitragin Co. (Milwaukee, Wisconsin) in a lyophilized form and maintained in the laboratory. It was reconstituted by breaking the ampoule and washing the powder into a YEM broth, maintaining aseptic conditions. The broth was incubated at 28°C on an Orbit

Envirocons Shaker (Lab-Line Inc.) and subcultured onto agar slants after it had developed turbidity on the fourteenth day. The cultures were allowed to grow for 10 days before storage at 4°C.

All the bacterial strains were subcultured every 2 months.

II.3. Characterization of bacterial strains.

The bacterial strains from nodules of *L. maritimus* and *O. campestris* were characterized using fast-growing *R. leguminosarum* biovar *vicia* and slow-growing *Bradyrhizobium* 32HI as reference strains.

II.3.1 Gram stain and Cell dimensions.

To determine bacterial cell shape and estimate cell dimensions, the Gram staining technique (Vincent 1970) was used. Smears were first made on clean, flamed and cooled microscopic slides from YEM broth cultures in the log phase. They were air-dried, heat-fixed onto the microscopic slides and stained with crystal violet solution for 1 min. This was followed by rinsing with water, flooding with iodine solution for 1 min and treating with 95% iodinated ethanol for 5 mins. The smears were washed with water, counterstained with safranin, rinsed with water and allowed to dry.

The smears were mounted with histoclad and observed at X1000 under oil immersion with a "Wild Heerburg" light microscope. For calculating the cell dimensions a standard calibrated ocular lens was used with the light microscope.

II.3.2. Motility test.

Motility of the various strains was assessed using the method described by Smibert and Krieg (1981). A semi-solid YEM medium (0.3% agar) was prepared and dispensed in 5 ml aliquots into 10 ml test tubes. A pin was used to stab 6-day old cultures into the semi-solid YEM, observing aseptic conditions. The tubes were incubated at 23-30°C for 6-12 days and observed for any migration from the point of inoculation.

II.3.3. Catalase activity.

2 drops of 3 to 6 day old bacteria suspensions were transferred with a loop onto glass slides and reacted with a drop of 1.5% hydrogen peroxide (Smibert and Krieg 1981). The various mixtures were observed for gas evolution, a test for positive catalase activity.

II.3.4. Growth patterns.

Growth of the bacterial strains on solid media was

investigated using the time taken for cells to form 1 mm diameter colonies as a measure of their growth rates. YEM agar plates were each inoculated with 1 ml of the diluted bacteria cell suspensions and inoculated at 23-30°C. Characteristics such as the first appearance and the morphology of the colonies were noted. The size of the colonies was measured daily until reaching 1 mm in diameter.

Generation time, which is the time taken for the doubling of the bacterial cells, was calculated for each isolate. Ten-ml portions of bacterial broth cultures were periodically removed, washed thoroughly in sterile physiological saline (0.85%, w/v, NaCl in distilled water) and resuspended in the saline solution to give the original volume. Optical density (OD) readings were taken using a Bausch and Lomb Spectronic 1001 spectrophotometer at regular intervals to determine when there was a steady increase in absorbance with time. This marks the beginning of exponential growth phase and 6 readings were taken at intervals of 2 hours after this point. The apparent OD readings were corrected to give the true OD using the formula described by Lawrence and Maier (1977).

The generation time for each isolate was calculated using the formula:

$$G = \frac{t \log 2}{\log OD_2 - \log OD_1}$$

G= generation time

OD₁ = optical density of bacterial cell suspension at time 1

OD₂ = optical density of bacterial cell suspension at time 2

t = final time - initial time

II.3.5. Effect of NaCl.

The tolerance of the isolates to the presence of NaCl was studied by amending YEM agar to give concentrations of 0.1, 1 and 2% NaCl w/v. The pre-sterilized YEM agar media were distributed in 20 ml portions into petri dishes maintaining aseptic conditions, allowed to set and stored for 2 days to check for contamination. 1 ml portions of the bacterial cell suspensions diluted to contain 10-90 cells ml⁻¹ (described in "Bacteria inocula preparation", p 42) were inoculated to the plates and incubated at 25°C. Daily measurements of the diameter of the colonies were made under a Bausch and Lomb stereomicroscope with a millimeter scale.

The number of days taken by the isolates to form 1 mm diameter colonies on each treatment was used as a measure of tolerance of the bacteria to that particular concentration of NaCl. Plates with the conventional amount of NaCl (0.1% w/v) were used as controls.

II.3.6. Effect of pH:

YEM agar media were amended with NaOH and HCl to give pH values of 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. A pH Meter Model 7 (Corning Scientific Instruments) was used to take pH readings. The media were sterilized and poured in 20-ml portions and stored for 2 days to check contamination. The plates were inoculated with 1-ml portions of bacterial cell suspensions diluted to contain 10-90 cells ml⁻¹ and incubated at 25°C. Influence of pH was determined by the number of days taken for the bacteria cells to form 1 mm diameter colonies on agar plates with various pH values.

II.3.7. Acid production:

Rhizobial isolates were tested for the production of acids as described by Norris (1965). YEM agar was amended with 0.5% alcoholic solution of bromothymol blue, sterilized and poured into petri dishes in 20-ml portions. They were stored for 2 days to check for contamination. The agar plates initially had a greenish-blue coloration.

The isolates and reference strains were streaked onto the plates, maintaining aseptic conditions and incubated at the appropriate temperatures. Colour changes on the plates were noted on the 5th day for fast-growing *Rhizobium* strains and on the 9th day for the slow-growing *Bradyrhizobium* strain. Plates

which were not inoculated with bacteria were used as controls.

To study the extent of acid or alkali-production by the isolates, 100-ml portions of YEM broth were distributed into 300 ml Erlenmeyer flasks and sterilized. Initial pH of the sterilized media was read using a pH meter. Flasks were inoculated with 1 ml portions of the bacteria cell suspensions at concentrations of $1-9 \times 10^8$ cell ml^{-1} . They were incubated at the appropriate temperatures on a rotary shaker operating at 125 revolutions per minute for 6-12 days. The final pHs of the broths were read on the pH meter.

II.3.8. Temperature tolerance:

Tolerance of *Rhizobium* strains to different temperatures was studied using the agar plate method (Caudry-Reznick et al., 1986). One-ml portions of rhizobial cell suspensions diluted to contain 10-90 cells ml^{-1} were inoculated on agar plates and incubated at temperatures of 5, 10, 15, 20, 25, 30 and 35°C.

The number of days taken to form 1 mm diameter colonies was used as a measure of tolerance of the strain to that particular temperature. Observations and measurements were carried out for a period of 45 days.

II.3.9. Utilization of carbohydrates and organic acids:

A basal medium consisting of the following chemicals in mg l⁻¹ of distilled water was prepared and sterilized (Antonou et al. 1984).

CaCl ₂ .2H ₂ O	= 50
MgSO ₄ .7H ₂ O	= 200
NaCl	= 100
(NH ₄) ₂ SO ₄	= 300
K ₂ HPO ₄	= 520
KH ₂ PO ₄	= 410
biotin	= 0.25
CoCl ₂ .6H ₂ O	= 4 x 10 ⁻³ ;
CuSO ₄ .5H ₂ O	= 8 x 10 ⁻³ ;
H ₂ MoO ₄	= 9 x 10 ⁻³ ;
MnCl ₂ .4H ₂ O	= 1.81
ZnSO ₄ .7H ₂ O	= 0.22.

Carbohydrates were incorporated into the basal medium to give a concentration of 1% (v/v) and organic acids incorporated to give concentrations of 2, 10 and 20 mM (v/v). All the carbon sources were filter-sterilized, using 0.22 um pore Nalgene filters (Nalgene Company, Rochester, New York), into the presterilized basal medium. The resulting media were poured into plates maintaining aseptic conditions.

Each plate was inoculated with 1-ml portions of bacterial inocula prepared from the isolates and reference strains and

diluted to contain 10-90 cells ml⁻¹. They were incubated at the appropriate temperatures and observed daily for growth and formation of colonies.

II.3.10. Antibiotic resistance tests:

Resistance of bacterial strains to certain antibiotics was investigated using the antibiotic susceptibility test disc technique (Au 1983, Barry and Thornsberry 1980).

Thick cell suspensions of the bacterial strains were spread on YEM agar plates with a bent glass rod observing aseptic conditions. This was done so that a thick uniform growth could be obtained on the plates. Susceptibility test discs impregnated with various antibiotics at specified concentrations (Oxoid Limited, Hampshire, England) were aseptically introduced, 3 to each inoculated agar plate. The plates were then incubated at 25-28°C and observed daily for the presence of an inhibition zone around the discs. Blank discs were used as control.

Presence or absence of an inhibition zone around a disc showed that the bacteria were susceptible or resistant, respectively, to the antibiotic impregnated in that disc. Diameter of inhibition zones was measured and compared to the standard developed for some microorganisms by Barry and Thornsberry (1980).

II.3.11. Bacterial inocula preparation:

Bacteria inocula of all the *Rhizobium* and *Bradyrhizobium* strains were prepared by using a sterile loop to transfer some bacteria from agar slant cultures into 300 ml cotton-plugged Erlenmeyer flasks containing 100 ml YEM broth. The flasks were incubated at 25°C and 28°C for the fast-growing *Rhizobium* strains and the slow-growing *Bradyrhizobium* strains respectively on the rotary shaker operating at 125 revolutions per minute. The broth cultures were harvested, at the log phase, by centrifuging at 10,000g and washing the pellets twice in a sterile saline solution and finally bringing them to the required concentration with a saline solution.

II.4. The plants:

II.4.1. Seed germination:

Seeds of *Oxytropis campestris*, *Pisum sativum* and *Vicia faba* were surface-sterilized by momentarily exposing them to 95% ethanol followed by 3-6 minutes immersion in 3% hydrogen peroxide. Seeds of *Lathyrus maritimus* and *Vicia cracca* showed dormancy which was broken by immersing them in concentrated sulphuric acid for 20-30 minutes. This method surface-sterilized them as well.

All the seeds were thoroughly rinsed in several changes

of sterile distilled water and transferred into pre-sterilized petri dishes containing moistened filter paper, maintaining aseptic conditions. They were placed in the dark for 6 days and the percentage germination determined as follows:

$$\frac{\text{Number of seeds that produced radicle}}{\text{Total number of seeds}} \times 100$$

II.4.2. Plant growth conditions and nodular characteristics:

Germinated seeds (as described in "Seed germination", p 42) of *L. maritimus* and *O. campestris* were planted in pots containing previously sterilized vermiculite. The seedlings were inoculated with the same volume of the appropriate inocula (described in "Bacteria inocula preparation" p 42) and grown in an environmental chamber with a regimen of 16 h light: 8 h dark at 16°C and 8°C respectively to simulate field conditions during the growing season. A nitrogen-free nutrient solution (Ellfolk 1960) was used to water the plants.

Roots were sampled to measure the nodules and study the external morphology, distribution and total number of nodules for each plant on 20, 40, 60, 80 and 100 days after inoculation.

Nodule morphology and distribution on roots of plant specimens collected from the field were also documented.

II.4.3. Estimation of nitrogen-fixation capacity of plants:

The method used to estimate the nitrogen fixation rates was modified from that of Hardy et al. (1968). For laboratory-grown plants, samples in 3 replicates were kept at the selected temperatures for a day. Their nodulated root systems were then placed in 13 ml vacutainer glass serum tubes (Becton, Dickerson and Co., Mississauga, Ontario). 0.4-ml of air was removed with a syringe and the same quantity of acetylene was introduced into each of the vacutainer tubes. They were incubated at 5, 10, 15, 20, 25, 30 and 35°C for 1 hour. With field-collected plants, samples of nodulated root systems in three replicates were incubated in 300 ml tubes with air-tight rubber stoppers at 20°C and 3 ml portions of air were replaced with acetylene.

Acetylene used in this experiment was generated in the laboratory by reacting calcium carbide with distilled water. The gas was collected from the reaction bottle through a rubber tubing into vacutainers under water.

Three-ml volumes of the gas mixture were drawn from the incubating vessels after the incubation period and injected into a gas chromatograph to assay for the reduction of acetylene to ethylene using a previously-described system (Boyle and Patriquin 1980). This consists of a model GC 9700 Basic Gas Chromatograph (Carle Instruments, Anaheim,

California) with a Poropak T column and a flame ionization detector which gives output to an Omniscribe recorder (model B 5118, Houston Instruments, Austin, Texas). Ethylene and the carrier gases consisting of helium, hydrogen and air were obtained from Canadian Liquid Air Company, St Johns, Newfoundland. The carrier gases had a flow rate of 20 ml/min. The column temperature was maintained at 50°C.

Ethylene peak heights for each treatment, which is a measure of the amount of ethylene present in the gas mixture injected, were measured directly from the resulting chromatogram. These were compared to a standard curve obtained by injecting a known volume of ethylene gas into the gas chromatograph. Total weight of nodules in each vacutainer was also measured. Rates of acetylene reduction were calculated as μ moles of ethylene produced per unit nodule fresh weight per unit time. Statistical tests were performed using the methods described by Bishop (1980) and Little and Hills (1978).

II.4.4. Nodulation patterns:

The isolates were tested for their ability to cross-infect their hosts or nodulate *P. sativum*, *V. cracca* and *V. faba*. Studies were done using 500 ml flasks and semi-solid nitrogen-free nutrient agar (Vincent 1970).

Composition of the medium used is (Jensen 1942):

CaHPO_4 = 1.0 g

K_2HPO_4 = 0.2 g

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ = 0.2 g

NaCl = 0.2 g

FeCl_3 = 0.1 g

Distilled H_2O = 1.0 l

Agar = 8.0 g

Trace elements (Gibson, 1963) were added as 1 ml/litre of a stock solution containing (values are in weight by volume of distilled H_2O):

Bo = 0.05%

Mn = 0.05%

Zn = 0.005%

Mo = 0.005%

Cu = 0.002%

The pH of the nutrient agar medium was adjusted to 6.8 and dispensed in 250-ml portions into the flasks, cotton-plugged and autoclaved. Germinated seedlings, (as described in "Seed germination", p 42) were transferred to the flasks containing slanted nutrient agar medium under aseptic conditions. Each flask was inoculated with 10 ml of the desired bacteria inoculum (described in "Bacteria inocula preparation", p 42). Uninoculated seedlings were used as controls.

The flasks containing the seedlings were then transferred

to growth cabinets with a regimen of 16 hours light and 8 hours dark at 23 and 12°C respectively. Nodulation tests were performed periodically and a positive result was recorded only if a rhizobial strain could be re-isolated from newly formed nodules and cultured on YEM agar plates and if it exhibited the same characteristics as the original strain.

II.5. Light and electron microscopical studies of nodule tissues:

Light and electron microscopical techniques were used to study the anatomy of nodules of both field-collected and laboratory-grown specimens of *L. maritimus*. Plants were sampled and shaken to get rid of excess soil or vermiculite. Nodulated root systems were immediately fixed in a mixture of gluteraldehyde and formalaldehyde (Karnovsky 1965). "Pink" nodules (effective because of the presence of leghemoglobin) were carefully selected from the nodulated root systems, dissected in a longitudinal plane with a blade and transferred into bottles containing fresh Karnovsky's fixative. They were kept at 4°C for 1 hour (Bal et al. 1989).

The nodule slices were washed in 5 changes of 0.1 M phosphate buffer (Sorensen's buffer) and subsequently kept in the buffer overnight to remove excess fixative. They were post-fixed with 1% osmium tetroxide in Sorensen's buffer for 1 hour in the dark and followed by washing with the buffer to

remove excess osmium tetroxide. The nodule slices were gradually dehydrated with an ethanol series of 50, 70, 80, 95 and 100%; 3 changes were made at the 100% ethanol concentration in 1 hour to ensure complete dehydration.

To localize lipid bodies in the nodule tissues, some of the samples were treated with 1% p-phenylenediamine in 70% ethanol for 1 hour after the 70% level in the ethanol series before being taken through the rest of the series (Bal 1990). To serve as control, some nodule slices were treated with hexane, which acts to extract lipids bodies, for an hour after dehydration (Bal 1990). This was followed by gradual rehydration through 100, 95, 80, 70 and 50% ethanol series and distilled water. These samples were again osmicated, dehydrated to 70% ethanol, treated with 1% p-phenylenediamine and then taken through the rest of the series as described above.

Completely dehydrated nodule samples were gradually embedded with Spurr's resin using graded mixtures of ethanol and resin (Spurr 1969). This was done by initially transferring them into bottles containing a mixture of 3 parts 100% ethanol and 1 part Spurr's resin, followed by 1 part 100% ethanol and 1 part Spurr's resin and then 1 part 100% ethanol and 3 parts Spurr's resin. Each treatment was carried out for an hour under vacuum. The samples were then transferred into pure Spurr's media and kept overnight under vacuum to facilitate complete replacement of ethanol by the embedding

media.

The nodule samples were embedded using flat rubber blocks with molds tapering at one end. The molds were filled with fresh Spurr's resin and the samples placed at the tapered tip with the desired orientation, polymerized in an oven maintained at 70°C.

Semi-thin and ultra-thin sections were made using a "Porter Blum" MTI ultramicrotome with glass knives. Sections of thickness 0.5-1.5 μm were picked onto glass slides, stained with toluidine blue and mounted with histoclad. Light micrographs were taken with Zeiss I photomicroscope.

For electron microscopy ultra-thin sections were routinely stained with uranyl acetate followed by lead citrate. They were allowed to dry in the air before mounting in a Zeiss 109 transmission electron microscope.

Area of lipid bodies and the total area the tissue regions were measured on light micrographs of nodule sections using a Carl Zeiss MOP 3 digital analyzer. The values obtained were expressed as the ratio of tissue region occupied by lipid bodies.

III. RESULTS

III.1.1. Field-collected plant samples:

The specimens of *L. maritimus* and *O. campestris* collected from the field were all found to be nodulated and the nodules were elongated, indeterminate and perennial. Table 4 gives the ranges of the length of nodules in the two species. *O. campestris* had a slender nodule structure and nodules of *L. maritimus* were mostly thickened below the meristematic region. Also, the older nodules of the two species, found near the tap roots, were mostly branched and the younger ones attached to the secondary roots were unbranched. They showed various degrees of branching with branching being more prominent in nodules of *O. campestris* than in those of *L. maritimus*.

The nodules of both species were nitrogen-fixing as shown by the acetylene reduction assay. Table 5 shows the rates of ethylene produced (acetylene reduced) per gram nodule fresh weight which is an indirect method of determining nitrogenase activity of the nodules. Nodules of *L. maritimus* showed a higher rate of nitrogenase activity than that of *O. campestris*.

Table 4: Length of nodules of field-collected plants.

Species	Length (mm)
<i>O. campestris</i> (n=72)	7.0 - 12.0
<i>L. maritimus</i> (n=75)	6.0 - 11.0

n is the number of nodules sampled from 3 plant specimens.

Table 5: Acetylene reduction assay (nitrogenase activity) of nodules of field-collected plant samples.

Species	Nitrogenase activity (μmol of ethylene produced/gram fresh weight/hour)		
	X	\pm	S.E.
<i>L. maritimus</i> (n=8)	0.674	\pm	0.077
<i>O. campestris</i> (n=9)	0.531	\pm	0.090

n is the number of plant specimens.

III.1.2. Seed germination:

Seeds of *O. campestris*, *P. sativum* and *V. faba* exhibited a high percentage of germination - 90 to 100%. Seeds of *L. maritimus* and *V. cracca*, on the other hand, did not germinate until they were treated with concentrated sulphuric acid. This treatment resulted in 85 and 95 percent germination in *L. maritimus* and *V. cracca* respectively.

III.1.3. Plant growth and nodule characteristics:

Plants of *O. campestris* and *L. maritimus* all formed nodules when they were inoculated with the original bacterial isolates and grown under laboratory conditions (Figures 4 and 5). Young nodules of both species were externally characterized by the presence of 2 colored regions; a distal white zone and a proximal pink zone. Root nodules were initially hemispherical in shape but became elongated with age. In some, their meristems divided with time to give rise to branched nodules.

Table 6 shows increases in length of nodules with time given in days after inoculation. Profound elongation and branching were observed in nodules of both species when they were sampled for observation on the 60th day after inoculation. Nodules of *O. campestris* grew to 4.0-5.5 mm in length and their branched lobes were slender, lying in a single plane and

creating a "fan-shaped palmate" structure described by Allen and Allen (1981) for some legume species. Nodules of *L. maritimus* were plump and grew to 4.0-5.0 mm in length. Their branching lobes lay in more than one plane.

Table 7 shows the mean number of nodules formed on plant species with time which is given in days after inoculation. For both *L. maritimus* and *O. campestris* the mean numbers of nodules increase from the 20th to the 40th day. There were no significant increases in nodule numbers after the 40th day.

Fig. 4. Photograph of laboratory-grown specimen of *L. maritimus* showing root nodules.

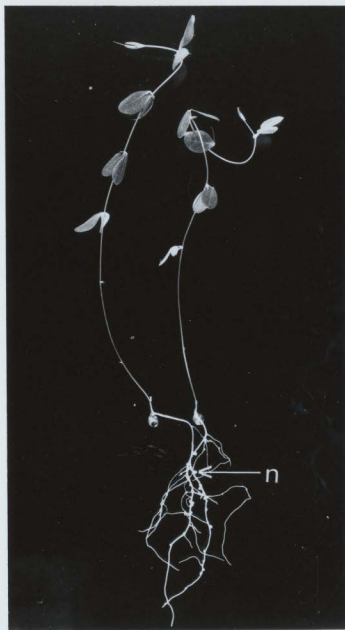


Fig. 5. Photograph of laboratory-grown specimen of *O. campestris* showing root nodules.

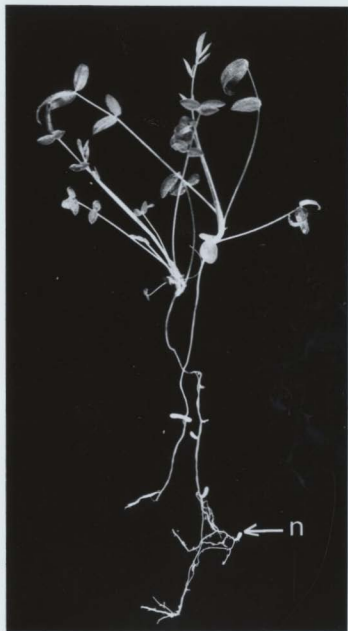


Table 6: Average increase in nodular length (mm) of laboratory-grown specimens with time (days after inoculation).

Days	<i>L. maritimus</i>	<i>O. campestris</i>
	(length of nodules in mm)	
	(n=9)	(n=9)
20	1.0-2.0	1.0-2.0
40	2.0-3.5	2.0-3.0
60	3.0-4.5	3.5-4.5
80	4.0-5.0	4.0-5.5
100	4.0-5.0	4.0-5.5

n is the number of plants from which nodules were sampled.

Table 7: Total number of nodules formed per plant with time (days after inoculation).

Days	<i>L. maritimus</i>	<i>O. campestris</i>
	(n=9)	(n=9)
20	25 ± 9	18 ± 8
40	34 ± 10	30 ± 11
60	33 ± 11	32 ± 10
80	33 ± 14	30 ± 12
100	34 ± 16	32 ± 14

n is the number of plant specimens.

III.1.4. Nitrogenase activity at various temperature regimens:

Table 8 gives rates of nitrogenase activity in *L. maritimus* and *O. campestris* at various temperatures as estimated by the acetylene reduction method.

Both plant species show highest nitrogenase activity at 20°C, however they were still capable of exhibiting activity at low temperatures. For *O. campestris*, the plants maintained 80% of their maximum nitrogenase activity at a temperature as low as 5°C but only 21% at a higher temperature of 30°C. *L. maritimus* plants maintained 35 and 39% of maximum activity at 5 and 30°C respectively.

Table 8. Acetylene reduction assay (nitrogenase activity) in laboratory-grown plants at various temperatures (values given as μmol of ethylene produced/gram nodule fresh weight/hour).

Temp. ($^{\circ}\text{C}$)	<i>L. maritimus</i>	<i>O. campestris</i>	<i>G. max</i> * -
	(n=9)	(n=9)	
5	2.141	7.403	-
10	2.909	7.513	29
15	4.779	8.480	86
20	6.034	9.250	214
25	4.450	7.807	176
30	3.696	4.571	250
35	3.373	3.000	151

LSD at 5% level 1.225 1.191

n is the number of plant specimens assayed.

* Data (in μmol of ethylene produced/ gram nodule fresh weight/ day) extracted from Hardy et al. (1968).

III.2. Characterization of rhizobial strains:

The results of some of the preliminary characterization of bacterial isolates are given in Table 9. The isolates were found to be gram negative rods, motile, acid-producing and exhibited catalase activity.

A characteristic of the rhizobial strain isolated from *L. maritimus* is the copious production of extracellular polysaccharides (ECP). It secreted EPC in quantities greater than those of the strains used in the studies. The rhizobial isolate of *O. campestris* produced ECP in quantities greater than the slow-growing reference strain but lesser than the fast-growing reference strain.

The mean generation time for the rhizobial isolates and the reference strains are given in Table 10. The isolate of *L. maritimus* had a generation time which was closer to the fast-growing strain. Isolate of *O. campestris* had a longer generation time.

Table 9: Results of preliminary characterization of the isolates.

	<i>Rhizobium</i> sp. (<i>L. maritimus</i>)	<i>Rhizobium</i> sp. (<i>O. campestris</i>)
Gram reaction	Gram -ve rods, some cocci forms found in young cultures.	Gram -ve rods, some cocci forms found in young cultures.
Cell dimensions	1.5-2.3 μm in length by 0.5- 0.8 μm in width.	1.5-2.0 μm in length by 0.5- 0.6 μm in width.
Motility	Positive results shown in the motility test.	Positive results shown in the motility test.
Catalase activity	Isolate exhibit catalase activity	Isolate exhibit catalase activity
Acid or alkaline production	Acid-producing, could reduce pH of growth medium.	Acid-producing, could reduce pH of growth medium.

Table 10. Comparison of growth patterns of rhizobial isolates and reference strains at optimum conditions.

Strain	Mean generation time (hours)
<i>Rhizobium</i> sp. (<i>L. maritimus</i>)	7.6
<i>Rhizobium</i> sp (<i>O. campestris</i>)	9.4
<i>Rhizobium legumi- nosarum</i> bv. <i>vicia</i>	6.1
<i>Bradyrhizobium</i> 32H1	15.2

III.2.1. Temperature effects:

The number of days taken to form 1 mm diameter colonies on solid media by the rhizobial isolates and the reference strains at various temperatures was used as a measure of their tolerance to those temperature conditions. Table 11 shows the values obtained for this experiment.

The isolates had the greatest growth rate, that is the lowest number of days taken to form 1 mm diameter colonies, at 23°C whilst the reference strains had theirs at 28°C. The isolates could grow at 5 and 10°C, though at a very slow rate. These low temperatures had the effect of completely inhibiting growth of the reference strains.

High temperatures slowed down the growth rate of the isolates.

Table 11: Growth of rhizobial isolates and some reference strains at different temperatures.

Strain	Number of days taken to form 1mm diameter colonies at the specified temperatures.			

	5°C	10°C	23°C	28°C
<i>Rhizobium</i> sp.				
(<i>I. maritimus</i>)	40	28	5	11
<i>Rhizobium</i> sp.				
(<i>O. campestris</i>)	36	24	6	13
<i>R. leguminosarum</i>				
biovar <i>vicia</i>	NG	NG	6	3
<i>Bradyrhizobium</i>				
32HI	NG	NG	12	8
NG - no growth				

III.2.2. Utilization of carbohydrates and organic acids:

Table 12 shows the relative abilities of the rhizobial isolates and reference strains to utilize various carbohydrates and organic acids as the sole source of carbon.

Rhizobium sp. (*L. maritimus*) like the fast-growing reference strain *R. leguminosarum* biovar *vicia* could utilize a wide range of carbohydrates and organic acids incorporated as sodium salts. For the carbohydrates, only D-maltose could not be utilized. For the organic acids, the isolate could not grow on agar media with D-lactate at all concentration levels, and oxaloacetate and α -ketoglutarate at lower concentrations. It could utilize citrate at lower concentrations but growth was totally inhibited at a high concentration level.

The isolate of *O. campestris* resembled the slow-growing reference strain *Bradyrhizobium* 32H1 in its ability to utilize various carbohydrates and organic acids because they both had a limited range.

Table 12 :Comparison of utilization of carbohydrates and organic acids by rhizobial isolates and 2 reference strains.

		<i>R. sp.</i> (<i>L. m.</i>)	<i>R. sp.</i> (<i>O. c.</i>)	<i>R. legumi-</i> <i>nosarum</i>	<i>B.</i> 32HI
Carbohydrate:					
(1%)		-----	-----	-----	-----
L-arabinose		+	+	+	+
Dulcitol		+	-	+	-
Fructose		+	+	+	+
D-Galactose		+	+	+	+
D-Glucose		+	+	+	+
Lactose		+	+	++	-
D-Maltose		-	-	+	-
D-Mannitol		++	++	++	++
Mannose		+	+	++	-
Raffinose		+	-	+	-
L-Rhamnose		+	+	+	-
Sucrose		+	-	+	-
D-Trehalose		+	-	+	-
D-Xylose		++	+	+	+
Organic acids (mM)					
Acetate	2	+	-	+	-
	10	+	+	+	+
	20	+	+	+	+
Citrate	2	+	+	+	-

Table 12 continued:

	10	+	+	+	+
	20	+	+	+	+
Glyoxylate	2	+	+	-	+
	10	+	+	+	+
	20	++	+	+	+
α -Keto-	2	-	-	+	+
glutarate	10	+	+	+	+
	20	+	+	+	+
D-lactate	2	-	-	-	-
	10	-	-	-	-
	20	-	-	-	-
L-malate	2	+	-	+	-
	10	+	-	+	-
	20	+	-	++	-
Oxalo-	2	-	-	-	-
acetate	10	-	-	-	-
	20	+	+	+	-
Pyruvate	2	+	-	+	-
	10	++	+	++	-
	20	++	+	++	+
Succinate	2	+	+	+	-
	10	++	+	++	-
	20	++	+	++	-

++ = excellent growth, + = good growth, - = no growth

III.2.3. Effect of pH:

Table 13 shows the effect of various pH levels on the growth of rhizobial isolates and the reference strains in growth media.

All the bacterial strains grew best at a pH of 6.8. *Rhizobium* sp. (*L. maritimus*) could tolerate a wide range of pH levels - from 4.5 to 9.0. *Rhizobium* sp. (*O. campestris*), like the fast-growing reference strain could not tolerate extreme pH levels. Their range of tolerance lay close to neutral pH, 5.8-8.0.

The slow-growing reference strain could only tolerate acidic pH levels and could not grow in alkaline growth media.

Table 13: Tolerance of rhizobial isolates and some reference strains to different pH levels in growth media.

Strain	pH values				
	4.5	5.8	6.8	8.0	9.0
<i>Rhizobium</i> sp.					
(<i>L. maritimus</i>)	+-	+	++	+	+-
<i>Rhizobium</i> sp.					
(<i>O. campestris</i>)	--	+-	++	+-	--
<i>R. leguminosarum</i>					
biovar <i>vicia</i>	--	+-	++	+-	--
<i>Bradyrhizobium</i>					
32HI	+-	+	++	--	--

++ = excellent growth

+ = good growth

+- = scanty growth

-- = no growth

III.2.4. Effect of NaCl concentration:

Table 14 shows the effect of NaCl concentration on the growth of the isolates and the reference strains.

All the bacterial strains had excellent growth at the normal NaCl used in growth media; i.e. 0.1%. *Rhizobium* sp. (*L. maritimus*) had good growth at 1% NaCl concentration and could still grow at the 2% level at a very slow rate.

Rhizobium sp. (*O. campestris*) and the fast-growing reference strain *R. leguminosarum* biovar *vicia* could rarely grow at a NaCl concentration of 1% and had no growth at the 2% level. Growth was completely suppressed in the slow-growing reference strain *Bradyrhizobium* 32HI at both 1% and 2% levels.

Table 14: Effect of NaCl concentration on growth of rhizobial isolates and reference strains.

Strain	NaCl concentration (%)		
	0.1	1	2
<i>Rhizobium</i> sp.			
(<i>L. maritimus</i>)	++	+	+-
<i>Rhizobium</i> sp.			
(<i>O. campestris</i>)	++	+-	--
<i>R. leguminosarum</i>			
biovar <i>vicia</i>	++	+-	--
<i>Bradyrhizobium</i> 32HI	++	--	--

++ = excellent growth

+ = good growth

+- = scanty growth

-- = no growth

III.2.5. Intrinsic antibiotic resistance tests:

Table 15 shows the results of intrinsic antibiotic resistance tests. *Rhizobium* sp. (*L. maritimus*) showed resistance to chloramphenicol, erythromycin, nalidixic acid and ampicillin at all disc concentration levels, and for tetracycline at a disc concentration of 5 µg. The highest and lowest susceptibilities were shown to kanamycin and neomycin respectively, both at disc concentration of 30 µg.

Rhizobium sp. (*O. campestris*) showed total resistance to only chloramphenicol. It showed resistance to erythromycin and tetracycline at a disc concentration of 5 µg. The highest susceptibility was shown to streptomycin at a disc concentration of 25 µg. The lowest susceptibility was shown to both erythromycin and kanamycin at disc concentration levels at 15 and 5 µg respectively. The table also shows the intrinsic resistance and susceptibility of the reference strains to the various antibiotics. The fast-growing *R. leguminosarum* biovar *vicia* was susceptible to all the antibiotics except ampicillin and nalidixic acid. The slow-growing *Bradyrhizobium* 32HI was on the other hand, resistant to all the antibiotics except streptomycin at all disc concentrations, and neomycin and kanamycin at a disc concentration of 30 µg.

Table 15: Intrinsic antibiotic resistance tests on rhizobial isolates and some reference strains.

Antibiotic ($\mu\text{g}/\text{disc}$)	<i>R. sp</i> (<i>L. m.</i>)	<i>R. sp</i> (<i>O. c.</i>)	<i>R. legumi-</i> <i>nosarum</i>	<i>B.</i> 32HI
-----	-----	-----	-----	-----
Chloram- (10)	+	+	18*	+
phenicol (30)	+	+	28	+
Erythro- (5)	+	+	7	+
mycin (15)	+	8	16	+
Tetra- (5)	+	+	12	+
cycline (30)	15	10	23	+
Ampi- (2)	+	16	+	+
cillin (10)	+	27	+	+
Strepto- (10)	17	21	17	22
mycin (25)	20	28	25	34
Nalidixic (30)	+	9	+	+
acid				
Neomycin (30)	13	15	12	19

Table 15 continued:

Kanamy-	(5)	20	8	21	+
cin	(30)	34	20	34	11

+ = resistant;

a = mean of diameters of inhibition zone (mm)

n = 6

III.3. Nodule anatomy

The overwintered nodule of *L. maritimus* revealed activation of the meristem and the formation of new nodule tissue which was colonized by rhizobia (Figure 6). This tissue was recognized as the invasion zone where infection threads could be seen along with the release of rhizobia (Figure 7). The nodules developed in the laboratory showed a clearly defined zonal differentiation from the tip to the point of attachment to the root (Figure 8). The infected cells of the nodules were enlarged compared to the uninfected cells (Figure 8a). The nodules were also found to contain transfer cells in the pericycle of the vascular bundles (Figure 9).

Lipid bodies and starch granules were found in some cells of the nodule tissue. The lipid bodies were stained by the pPD method (Figure 10). Control preparations treated with hexane resulted in the extraction of lipids from the lipid bodies, which appeared as "lipid body ghosts" (Figure 11).

Figure 6. Light micrograph of a section of field-collected nodule of *L. maritimus* showing the formation of new rhizobia-infected tissue, the invasion zone (iz) and the senescent dormant tissue (st) from the previous year, X 300.

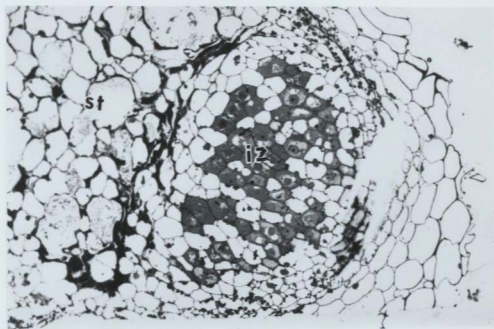


Figure 7. Light micrograph of the invasion zone of a field-collected nodule under higher magnification showing the infection thread (it) and release of rhizobia (arrows), X 1200.

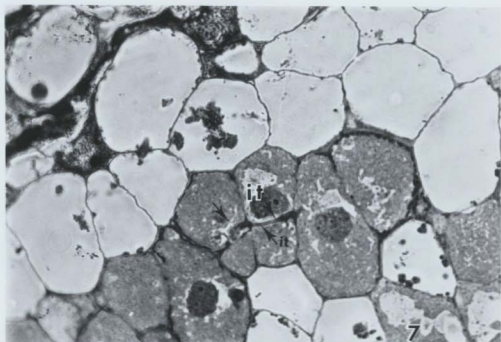


Figure 8. Light micrograph of a longitudinal section of the indeterminate nodule of laboratory-grown specimen of *L. maritimus* showing the apical meristem (am), the invasion zone (iz), the early symbiotic zone (esz), the late symbiotic zone (lsz) and the vascular elements, X 120.

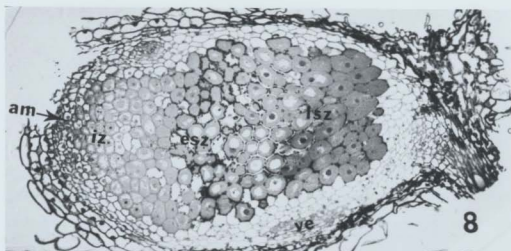


Figure 8a. Light micrograph of the symbiotic zone under higher magnification showing the enlarged infected cells and the uninfected interstitial cells, X 1200.

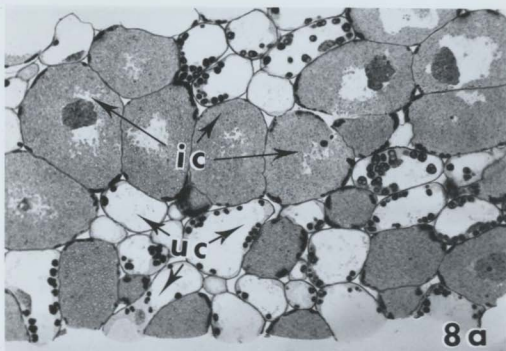


Figure 9. Electron micrograph showing parts of transfer cells within the vascular elements. Note the ingrowths of the cell wall (arrows), X 28,000.

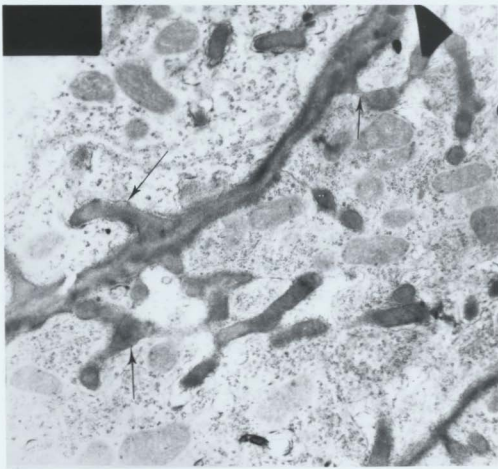


Figure 10. Electron micrograph of a nodule section of *L. maritimus* showing pPD-stained lipid bodies (arrows) in (A) infected cells and (B) uninfected cells, X 28,000.

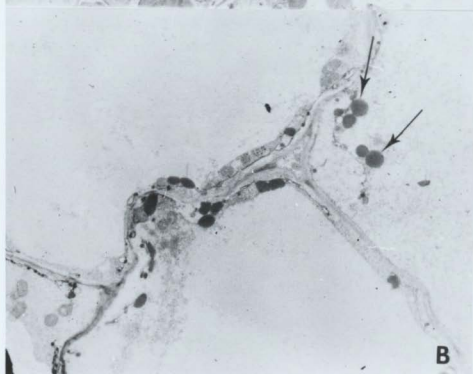


Figure 11. Electron micrograph of hexane-treated nodule section of *L. maritimus*. Note the "lipid body ghosts" (arrows) in (C) infected cells and (D) uninfected cells, X 28,000.



III.3.1. Distribution of lipid bodies:

Table 13 gives the distribution of lipid bodies in the various regions of the nodule tissue as studied with light micrographs. Values are given as the percentage of area of the nodule tissue that is occupied by lipid bodies. The values show that lipid bodies are present as a high proportion of the cortical cells and the early symbiotic zone. The percentage area covered by lipid bodies is very low in the late symbiotic zone but has a higher value in the senescent region.

III.4. Cross inoculation tests:

The isolates from the two hosts failed to cross infect their hosts. Further cross inoculation tests performed showed that the isolate of *L. maritimus* could infect only *V. cracca* but not *Vicia faba* and *Pisum sativum* seedlings. The strain isolated from *O. campestris* could not infect any other legume host.

Table 16: Distribution of lipid bodies in various regions of nodules of *L. maritimus*.

Region	% of tissue area covered by lipid bodies
Cortical cells (n=24)	2.77 \pm 1.13
Early symbiotic zone (n=24)	3.37 \pm 1.02
Late symbiotic zone (n=24)	0.06 \pm 0.02
Senescent region (n=28)	1.74 \pm 0.92

n is the number of unit areas on the light micrographs of nodule tissue.

IV. DISCUSSION

IV.1. Bacterial characterization:

The two isolates generally exhibited the characteristics of *Rhizobium* listed by Jordan (1984); that is, the Gram reaction, cell dimensions, motility and ability to produce acid in YEM growth media. The isolate of *L. maritimus* closely resembled the reference strain *R. leguminosarum* biovar *vicia* in various aspects than did the isolate of *O. campestris*. The isolate *L. maritimus* also resembled the isolate of *V. cracca* previously characterized by Niles (1983). The resemblance reflects the close relationship they have, both belonging to the so-called "pea group" (Lim and Burton 1982, Elkan 1984). Their original hosts could be infected by the same strain of *Rhizobium*. They differed markedly, however, in other specific characteristics like effect of temperature, NaCl and different pH levels. These differences are discussed fully in IV.1.1., IV.1.2. and IV.1.3.

The isolate of *O. campestris* did not show a particularly close relationship with any of the reference strains. This agrees with the report by Prévost et al. (1987) which found that characteristics of rhizobia isolated from some arctic species of *Oxytropis* indicated that they have affinities with both the fast-growing and the slow-growing root nodule bacteria and suggested that the rhizobial strains have co-

evolved with their hosts. Schulman et al. (1988), also working with some arctic species of *Oxytropis*, have suggested that the rhizobia involved in symbiotic associations with arctic legumes may belong to a single species.

IV.1.1. Influence of temperature:

Although *Rhizobium* and *Bradyrhizobium* strains have a wide range of temperature tolerance (Jordan 1984) growth at low temperatures is rare (Trinick 1982, Jordan 1984). However Prévost et al. (1987) reported very good growth at low temperatures in most of the strains of *Rhizobium* isolated from three legumes indigenous to the Canadian high arctic which is similar to the findings of this investigation. There was good growth in the isolates at 5 and 10°C, unlike the reference strains, reflecting their adaptation to cold conditions. This should be expected because the isolates have to survive a long period of low temperature conditions every year. This again agrees with an earlier report by Ek-Jander and Fahraeus (1971) which indicated that clover rhizobia isolated from the subarctic region in Scandinavia showed a better adaptation to low temperature and grew faster at 10°C than isolates from southern areas.

The results of this investigation strengthens the conclusion made by Caudry-Reznick et al. (1986), working with isolates from some species of *Oxytropis*, that the *Rhizobium*

strains were well-adapted to survival and growth at low temperature conditions.

IV.1.2. NaCl tolerance:

Fast-growing *Rhizobium* strains have been reported to be generally less tolerant to a high concentration of NaCl while the slow-growing *Bradyrhizobium* strains are more tolerant (Elkan 1984). Although the isolate of *L. maritimus* exhibits fast-growing characteristics it is still able to tolerate a high concentration of the salt. This tolerance suggests an adaptive feature which might have been evolved by the bacteria because they are exposed to high concentration of salt. It grows and survives together with the host near the sea-shore and is therefore exposed to seawater. Adaptation to a high concentration of NaCl has also been reported in some strains of the pea group (Steinborn and Roughley, 1974).

Lindstrom and Lehtomaki (1988) found that some fast-growing *Rhizobium* could tolerate twice the concentration of salt previously reported to be the level of tolerance. Padmanabhan et al. (1990) have concluded that NaCl tolerance is extremely variable.

The isolate of *O. campestris* however had the same level of NaCl tolerance as the slow-growing *Bradyrhizobium* strain and could be put in that group on that basis.

IV.1.3. Tolerance to different pH levels:

The pH range tolerated by the isolates agrees well with that listed by Jordan (1984) which is generally between 4.5-9.5. The optimum pH level for growth varies among various strains. However, the greater overall sensitivity of fast-growing *Rhizobium* and tolerance of slow-growing *Bradyrhizobium* to low pH have often been reported (Parker and Graham 1964, Okafor and Alexander 1975, Munns 1977, Barimah-Asare et al. 1990).

The isolate of *O. campestris* resembled the slow-growing reference strain in its tolerance to pH. The isolate of *L. maritimus* had a wide pH range where growth could occur which is a very important adaptive feature. Munns (1977) concluded that it is most likely that pH interacts with other factors and therefore results of experiments on pH requirement or tolerance should be interpreted with care. In this investigation, since the routine growth medium was always used and all other conditions other than the hydrogen ion concentration were maintained constant, other factors did not have an influence on the results obtained.

IV.1.4. Carbohydrate and organic acid utilization:

With respect to carbohydrate and organic acid utilization, the two isolates resembled the fast-growing

reference strain more than the slow-growing reference strain. The carbohydrate utilization pattern exhibited by the isolates resembled that of temperate fast-growing rhizobia reported by Graham (1964) and Parker and Graham (1964). In agreement with other findings, the strains exhibiting mostly fast-growing characteristics, utilized a greater number of carbohydrates and organic acids. The same pattern of carbohydrate and organic acid utilization was obtained in the case of the isolate of *O. campestris* like that documented for some strains of arctic *Rhizobium* (Prévost et al. 1987).

The results agree with the findings of Padmanabhan et al. (1990) that fructose, galactose and mannose are easily utilized by most *Rhizobium* and *Bradyrhizobium* strains and that strains that metabolized fewer carbohydrates tended not to grow on disaccharides and polysaccharides like raffinose, lactose, and trehalose.

IV.1.5. Antibiotic resistance tests

With the intrinsic antibiotic resistance tests, the results show that the isolates cannot be classified either as slow-growing or fast-growing based on the generalizations made by Jordan (1984) in Bergey's Manual of Determinative Bacteriology. Jordan (1984) reported that spontaneous mutations to most antibiotics are a common component in "wild-type" or "native" strains explaining why the isolates could

not be characterized in general terms by these tests.

IV.2. External nodule characteristics:

The plant species used in this investigation have nodules which are described as perennial (Bergersen 1982). This type of nodule was characterized by the growth and development of new tissues during the beginning of each growing season (Figure 6). The result was the beaded and branching structure possessed by nodules of field-collected specimens. Field-collected nodules had active pink-coloured (presence of leghaemoglobin) distal portions where nitrogen-fixation took place and senescent proximal tissue where the symbiotic bacteroids had disintegrated (Figure 6).

The nodules which developed on roots of laboratory-grown plants increased in length up to a certain point and showed no further changes (Table 6). Continuous maintenance of the plant specimens under optimal growth conditions after this point did not cause further increases in the average size of the nodule. This suggests that the plant species have a particular maximum size of nodule growth for each season.

The nodules are adapted to overwintering and activation of the apical meristem for the formation of new tissues during the growing season (Figure 6). This accounts for the large differences in the nodule size and branching of field-collected specimens as compared to the maximum size and

structure of the nodules developed on laboratory-grown specimens (Table 4 and 6).

IV.3. Nitrogenase activity:

The difference in the values for the acetylene reduction assay of field-collected samples may not be a reflection of superior nitrogenase activity in *L. maritimus* compared with *O. campestris* but, rather, may be due to the fact that the samples assayed were of different ages and therefore may have contained different amounts of active nitrogen-fixing tissue per total weight of nodule tissue.

The same can also be said of the wide difference between the values for the acetylene reduction assay of field-collected and laboratory-grown samples. Plant specimens collected from the field must have gone through more than one growing season, resulting in the nodules containing more senescent tissue and hence lower nitrogenase activity per unit weight. On the other hand, laboratory-grown plants of each of the species show higher activities because they have nodules with mainly active nitrogen-fixing tissues. This should be expected because, according to Bergersen (1982), the relative volume of the active nitrogen-fixing tissue in the nodule determines the amount of nitrogen that is fixed by the nodule. To minimize the effect of differences in the amount of dormant senescent tissue on the estimation of nitrogenase activity,

Schulman et al. (1988) used the total number of meristems as a unit in quantifying nitrogenase activity in some perennial arctic legumes.

The ability of *L. maritimus* and *O. campestris* to reduce acetylene (nitrogenase activity) at low temperatures (Table 8) reflects adaptation of the plants and their symbionts to their environment (Barimah-Asare et al. submitted), a region where environmental temperatures are generally low for most of the growing season (Banfield 1981). Plant specimens of *O. campestris* had higher acetylene-reduction rates (nitrogenase activity) than those of *L. maritimus* at low temperatures suggesting a better adaptation to nitrogen fixation in the cold. This better adaptation may probably be due to the fact that *O. campestris* was collected in an arctic-alpine region. Arctic-alpine regions usually have ambient temperatures much lower than those at lower plains (Mani 1980).

Minchin et al. (1986) have criticized the acetylene reduction assay, using a closed system, to measure nitrogenase activity and have suggested the use of a continuous gas flow system. They also stated that the use of detopped/shaken roots to measure nitrogenase activity can produce substantial errors in the acetylene reduction assay which makes the assay invalid even when used for comparative purposes.

In this investigation the method of acetylene reduction assay in closed vessels was used to estimate nitrogenase activity in the plant specimens. Because all of the plant

specimens were subjected to the same experimental treatments, inherent errors, if any, were also carried through the experimental set-ups. Furthermore the experiments were repeated several times and gave consistent results. Analysis of variance tests performed on the results showed no significant differences (Table 8).

Saito et al. (1980) reported that there was no significant difference in acetylene reduction between the three systems studied - nodulated roots, disturbed whole plants and intact plants - during the first hour of measurement. They concluded that the results obtained with nodulated roots were comparable to and representative of intact plants. Also, according to Trinick et al. (1976), the disturbance of the plant, that is using detopped/shaken nodulated root systems, had no effect on the reduction of acetylene. Therefore the values obtained for the subsequent measurement of ethylene production after removal of the shoots represented that of the intact undisturbed plant.

The results of the experiments by Minchin et al. (1986) using the continuous gas flow system show that decreasing the temperature from 25 to 15°C, at which detopped/shaken nodulated root systems of soya bean were equilibrated and assayed, increased nitrogenase activity. This is in sharp contrast with the report by Hardy et al. (1968) who also used detopped/shaken nodulated root systems of soya bean. They found that decreasing the temperature from the optimum (25°C)

to 15°C caused a lowering in nitrogenase activity. In this investigation, using nodulated root systems, it was also found that a decrease in temperature below the optimum (20°C) caused a decrease in nitrogenase activity. These results further agree with the findings of Trinick *et al.* (1976) using nodulated root systems to study the effect of temperature on nitrogenase activity in some species of *Lupinus*.

IV.4. Nodule anatomy:

There have already been reports on the nodule anatomy of some species of *Oxytropis*, *O. maydelliana* and *O. arctobia* (Newcomb and Wood 1986, Prévost and Bal 1989). Therefore emphasis was given to the anatomical studies of nodules of *L. maritimus* in this investigation.

IV.4.1. Meristematic region:

Figure 8 shows that the meristem of *L. maritimus* consisted of apically-located, cap-shaped band of small cells. The cells were isodiametric as reported for some perennial nodules (Newcomb 1976, Newcomb and Wood 1986).

IV.4.2. Symbiotic region:

The infected zone consisted of cells that were enlarged

compared to the other cells of the nodule tissue (Figure 8). This zone had three structurally distinct areas - (a) the invasion zone with cells, recently derived from the meristem, having lipid bodies and some of the cells becoming invaded by infection threads (Figure 7), (b) the early symbiotic zone which was characterized by the presence of lipid bodies and high quantities of amyloplasts, (c) the late symbiotic zone with lesser quantities of lipids and amyloplasts.

The presence of a substantial number of lipid bodies (oleosomes) in the uninfected, newly-infected and cortical cells of root nodules of *L. maritimus* is particularly interesting. In plants, lipid bodies are mostly found in storage organs like seeds. However, they have been reported to be present in root nodules of peanut (Emerson and Bal 1988, Bal et al. 1989, Jayaram and Bal 1991) and some arctic legumes like *Oxytropis* sp. and *Astragalus* sp. (Newcomb and Wood 1986, Prévost and Bal 1989).

The functional role of lipid bodies in nodules is not fully understood but in the peanut they have been reported to be involved in nitrogen fixation being a supplementary source of energy (Siddique and Bal 1991, Bal and Siddique 1991). In arctic legumes lipids may be involved in protecting the host tissues from low temperatures, and therefore may control the ability of plants to survive exposure to temperature extremes (Sommerville and Browse, 1991). Newcomb and Wood (1986) reported that lipid bodies may provide a high energy source

for the process of nitrogen fixation by the bacteria in the infected cells of root nodules of arctic legumes. They further suggested that the presence of large amounts of lipid and starch may indicate a ready supply of stored energy so that fixation occurs without inhibition during the limited time in which soil temperatures allow growth and also represent some mechanism for increasing the temperature within the cells to facilitate growth and development.

L. maritimus is native to regions where the growing seasons are short and characterized by low temperatures. It is therefore reasonable to suggest that lipid bodies and starch granules play a role similar to that suggested for arctic legumes. The relative distribution of these lipid bodies (Table 16) and starch granules indicates that they may be utilized as a source of energy by the bacteroids for nitrogen fixation. This is because higher quantities of lipid bodies were found in the cortical tissue where there is no fixation and in the invasion zone where bacteroids are being formed and nitrogen is being fixed at comparatively lower rates. On the other hand lipid bodies are found in low quantities in the symbiotic zone which has fully developed bacteroids fixing nitrogen at higher rates.

Lipid bodies may also provide a ready source of molecular lipid necessary for the rapid proliferation of membrane materials which accompany the infection process (Newcomb and Wood 1986, Bal and Barimah-Asare 1991).

IV.4.3. Senescent region:

Because they displayed indeterminate growth pattern, mature nodules of *L. maritimus* had the older regions near the point of attachment to the root consisting of senescent dormant tissue. This zone consisted of cells with decayed bacteroids and had lost their structural features, unlike the infected zone. The number of lipid bodies increased in this region compared to that in the late symbiotic zone which may possibly be due to lipid accumulation originating from membranes of degenerating structures in the cells.

According to Bergersen (1982), in perennial nodules, some bacterial cells enter the senescent cells from remnants of infection threads. He has suggested that senesced tissue may serve as a reservoir of infection from which new nodule growth arises after overwintering.

IV.4.4. Transfer cells:

Transfer cells observed in nodules of *L. maritimus* have also been reported in the nodules of some species of Leguminosae (Newcomb and Peterson 1979, Pate et al. 1969). Pate et al. (1969) found that root nodules of non-legumes, as well as non-nitrogen-fixing root nodules of many genera of Leguminosae, appeared to lack these transfer cells. They therefore suggested that transfer cells are correlated with

nitrogen fixation. Newcomb and Peterson (1979) however, reported transfer cells in some ineffective nodules of *Pisum sativum* and also transfer cells located near nodules of *Glycine max* were found to develop before the onset of nitrogen fixation. This led them to suggest that the occurrence of transfer cells is independent of nitrogen fixation. The role played by transfer cells in nodules is therefore not clear.

CONCLUSIONS:

1. *L. maritimus* and *O. campestris* have the ability to exhibit nitrogenase activity at appreciable levels in the cold.
2. The isolate of *L. maritimus* is a fast-growing *Rhizobium* strain. The isolate of *O. campestris*, however, exhibited characteristics resembling both fast-growing *Rhizobium* and the slow-growing *Bradyrhizobium* species.
3. The isolates of *L. maritimus* and *O. campestris* tolerate low temperatures and the former further tolerate a wide pH range and a high NaCl concentration.
4. *L. maritimus* has nodules with an internal structure similar to that of previously-described indeterminate nodules. The nodules also possess lipid bodies.

REFERENCES

- Alexander, V., M. Billington and D. M. Schell, (1978): Nitrogen fixation in arctic and alpine tundra. In: Vegetation and Production Ecology of An Alaskan Arctic Tundra, (L. L. Tieszen, ed.), pp 539-558. Springer-Verlag, New York.
- Allen, O. N. and E. K. Allen, (1981): The Leguminosae. University of Wisconsin Press, Madison.
- Allen, O. N. and E. K. Allen, (1954): Morphogenesis of the leguminous root nodule. In: Abnormal and Pathological Plant Growth. Brookhaven Symposia in Biology, # 6 pp 209-234. Brookhaven National Laboratory, U.S.A.
- Antoun, H., L. M. Bordeleau and R. Savegeau, (1984): Utilization of the tricarboxylic acid intermediates and symbiotic effectiveness in *Rhizobium meliloti*. Plant and Soil, 77: 29-38.
- Au, W. Y., (1983): Interpretation of Susceptibility Zones in Antibiotic Tests used for Taxonomy of Marine Bacteria. B.Sc (Honours) Dissertation. Memorial University of Newfoundland, St. John's, Newfoundland.

Bal, A. K., (1990): Localization of plant lipids for light microscopy using p-Phenylenediamine in tissues of *Arachis hypogaea* (L). Stain Technology, 65 (2): 91-94.

Bal, A. K. and A. B. M. Siddique, (1991): Fine structural changes in peanut root nodules induced by nod⁺ fix⁻ strains of *Bradyrhizobium* with special reference to lipid bodies. Annals of Botany, 67: 309-315.

Bal, A. K. and J. Barimah-Asare (1991): Lipid bodies in rhizobia-infected root nodules of beach pea (*Lathyrus maritimus*). Proceedings of the Canadian Society of Microbiologists Annual Meeting, pp 78, London, Ontario.

Bal, A. K., S. Hameed and S. Jayaram, (1989): Ultrastructural characteristics of the host-symbiont interface in nitrogen-fixing peanut nodules. Protoplasma, 150: 19-26.

Banfield, C. E., (1981): The climatic environment of Newfoundland. In: The Natural Environment of Newfoundland - Past and Present, (A. G. Macpherson and J. B. Macpherson, eds), pp 83-153. Memorial University Printing Services, Newfoundland.

Barimah-Asare, J., P. J. Scott and A. K. Bal, (1990): Legume-

Rhizobium symbiosis in *Lathyrus maritimus*. Proceedings of the Annual Canadian Society of Microbiologists Meeting, pp 41, Calgary, Alberta.

Barimah-Asare, J., P. J. Scott and A. K. Bal, (submitted): Nitrogen fixation in 3 Newfoundland species of Leguminosae. Nordic Journal of Botany.

Barry, A. L. and C. Thornsberry, (1980): Susceptibility testing: Diffusion test procedures. In: Manual of Clinical Microbiology, Third edition, (E. H. Lennette, A. Boloros, W. J. Hausler Jr. and J. P. Truant, eds.), pp 463-474. American Society of Microbiology, Washington D.C.

Bergman, K., M. Gulash-Hofee, R. E. Hovestadt, R. C. Larosiliere, P. G. Ronco and L. Su, (1988). Physiology of behavioural mutants of *Rhizobium meliloti*: evidence for a dual chemotaxis pathway. Journal of Bacteriology, 170: 3249-3254.

Bergersen, F. G., (1980): Measurement of nitrogen fixation by direct means. In: Methods for Evaluating Biological Nitrogen Fixation. (F. G. Bergersen, ed.) John Wiley and Sons, Toronto.

Bergersen, F. G., (1982): Root nodules of legumes: Structure and Function. pp 23-43. Research Studies Press, Winchester.

Bergersen, F. G. and D. J. Goodchild (1973): Aeration pathways in soybeans root nodules. Australian Journal of Biological Sciences, 26: 729-740.

Bieberdorf, F. W., (1938): The cytology and histology of the root nodules of some leguminosae. Journal of the American Society of Agronomy, 30: 375-389.

Bishop, O. N., (1980): Statistics for Biology: A Practical Guide for the Experimental Biologist, 3rd edition. Longman Group Ltd., Essex.

Böcher, T. W., K. Holmen and K. Jakobsen, (1968): The Flora of Greenland. P. Haase and Son Publishers, Copenhagen.

Boyle, D. C. and D. G. Patriquin, (1980): Endorhizal and exorhizal acetylene-reducing activity in a grass (*Spartina alterniflora* L.)- diazotroph association. Plant Physiology, 66: 276-280.

Bulen, W. A., J. R. LeComte, R. C. Burns and J. Hinkson, (1965): Nitrogen-fixation studies with aerobic and photosynthetic bacteria. In: Non-heme Iron Proteins Role in Energy Conversion, (San Pietro, A., ed) pp 261-274. The Antioch Press, Yellow Springs, Ohio.

Burns, R. C. and R. W. F. Hardy, (1975): Nitrogen Fixation in Higher Plants. Springer-Verlag, New York.

Burris, R. H., (1977): Overview of nitrogen fixation. In: Genetic Engineering for Nitrogen Fixation, (A. Hollaender, ed.), pp 9-18. Plenum Press, New York.

Burris, R. H. and P. W. Wilson, (1957): Methods for measurement of nitrogen fixation. In: Methods in Enzymology, (S. P. Colowick and N. O. Kaplan, eds.) Vol 4, pp 355-367. Academic Press, New York.

Bushnell, O. A. and W. B. Sarles, (1937): Studies on the root-nodule bacteria of wild leguminous plants in Wisconsin. Soil Science, 44 (6): 409-423.

Caetano-Anollés, G., D. K. Crist-Estes and W. D. Bauer, (1988). Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. Journal of Bacteriology, 170: 3164-3169.

Carrol, W. R., (1934): A study of *Rhizobium* species in relation tonodule formation on the roots of Florida legumes. Soil Science, 37: 117-135.

Caudry-Reznick, S., D. Prevost and H. M. Schulman, (1986):

Some properties of arctic rhizobia. Archives of Microbiology, 146: 12-18.

Chandler, M. R. (1978): Some observations on infection of *Arachis hypogaea* (L) by *Rhizobium*. Journal of Experimental Botany, 26 (110): 749-755.

Cole, M. A. and G. H. Elkan, (1979): Multiple antibiotic resistance in *Rhizobium japonicum*. Applied and Environmental Microbiology, 37: 867-870.

Conklin, M. E., (1936): Studies of the root nodule organisms of certain wild legumes. Soil Science, 41: 167-185.

Cowan, S. T., (1974): Cowan and Steel's Manual for the Identification of Medical Bacteria 2nd edition. Cambridge University Press, London.

Dangeard, M. P. A., (1926): Recherche sur les tubercules radicaux des legumineuses. Le Botaniste, 26: 1-27.

Dart, P., (1977): Infection and development of leguminous root nodules. In: A Treatise on Dinitrogen Fixation, Section III, Biology, (Hardy, R. W. F. and Silver, W. S., eds) pp 367-472. John Wiley and Sons, New York.

Diaz, C. L., L. S. Melchers, P. J. J. Hooykaas, B. J. J. Lugtenberg and J. W. Kijne, (1989): Root lectin as a molecular determinant of host plant specificity in the *Rhizobium*-legume symbiosis. *Nature*, 338: 579-581.

Dilworth M. J., (1966): Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. *Biochemica Biophysica Acta*, 127: 285-294.

Dilworth, M. J., D. Subramanian, T. O. Munson and R. H. Burris, (1965): The adenosine triphosphate requirement for nitrogen fixation in cell-free extracts of *Clostridium pasteurianum*. *Biochemica Biophysica Acta*, 99: 486-503.

Dixon R. O. D. and C. T. Wheeler, (1986): Nitrogen Fixation in Plants, pp 45-60. Blackie, London.

Ek-Jander, J. and G. Fahraeus, (1971): Adaptation of *Rhizobium* to subarctic environment in Scandinavia. *Plant and Soil Special volume*, 129-137.

Elkan, H. G., (1981): The taxonomy of the Rhizobiaceae. In: *Biology of the Rhizobiaceae* (Giles, K. L. and Atherly A. G., eds), pp 1-14 Academic Press, Toronto.

Elkan, G. H., (1984): Taxonomy and metabolism of *Rhizobium* and its genetic relationships. In: Biological Nitrogen Fixation-Ecology, Technology and Physiology, (Alexander, M., ed.), pp 1-38. Plenum Press, New York.

Ellfolk, N., (1960): Crystalline leghemoglobin: purification procedure. *Acta Chemica Scandinavia*, 14: 609-616.

Emerson, C. J. and A. K. Bal, (1988): An SEM/TEM study of structures associated with bacteroids in peanut (*Arachis hypogaea*) root nodules. Proceedings of the 46th Annual Meeting of the Electron Microscopy Society of America, 288-289.

Evans, H. J. and L. E. Barber, (1977): Biological nitrogen fixation for and fibre. *Science*, 197: 332-339.

Evans, H. J. and S. A. Russell, (1971): Physiological chemistry of symbiotic nitrogen fixation by legumes. In: The Chemistry and Biochemistry of Nitrogen Fixation, (J. R. Postgate, ed.) pp 191-244. Plenum Press, London.

Fernald, M. L. (1950): Gray's Manual of Botany. 8th ed. American Book Co., New York.

Fred, E. B., I. L. Baldwin and E. McCoy, (1932): The Root Nodule Bacteria and Leguminous Plants. University of

Winsconsin Studies in Science, # 5, Madison.

Gallon, J. R. and A. E. Chaplin, (1987): An Introduction to Nitrogen Fixation. Cassel, London.

Gibson, A. H., (1963): Physical environment and symbiotic nitrogen fixation. I. The effect of recently nodulated *Trifolium subterraneum* L. plants. Australian Journal of Biological Science, 16: 28-42.

Gibson, A. H., W. R. Scowcroft, J. J. Child and J. D. Pagan, (1976): Nitrogenase activity in cultured *Rhizobium* sp. 32H1. Archives of Microbiology, 108: 45-54.

Graham, P. H., (1964): Studies on the utilization of carbohydrates and Kreb's cycle intermediates by Rhizobia using an agar plate method. Antonie Van Leeuwenhoek 30: 68-72.

Graham, P. H., (1976): Identification and classification of root nodule bacteria. In: Symbiotic Nitrogen Fixation in Plants, (P. S. Nutman, ed.) I.B.P. Handbook 7, Cambridge University Press, Cambridge.

Gunning, B. E. S., (1977): Transfer cells and their roles in the transport of solutes in plants. Science Prog. Oxford, 64: 539-569.

Hardy, R. W. F., R. D. Holsten, E. K. Jackson, and R. C. Burns, (1968): The acetylene-ethylene assay for nitrogen fixation. *Plant Physiology*, 43 (8): 1185-1207.

Hardy, R. W. F., R. C. Burns, and R. D. Holsten, (1973): Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biology and Biochemistry*, 5: 47-81.

Hitchcock, C. L., (1952): A revision of the North American species of *Lathyrus*. University of Washington Publications, Biology 15: 1-104.

Ingwersen, W., (1958): Manual of Alpine Plants. Will Ingwersen and Dunnsprint Ltd., Eastbourne, England.

Jayaram, S. and A. K. Bal, (1991): Cell biology of nitrogen-fixing peanut root nodules. *Plant, Cell and Environment*, (In Press).

Jensen, H. L., (1942): Nitrogen fixation in leguminous plants. I. General characters of root-nodule bacteria isolated from species of *Medicago* and *Trifolium* in Australia. *Proceedings of Linn. Society*, 66: 98-108.

Jordan, D. C., (1984): Family III; The Rhizobiaceae. In *Bergey's Manual of Systematic Bacteriology*, 1st edition, pp

234-248. Williams and Wilkins, Baltimore.

Jordan, D. C. and D. N. Allen, (1974): Family III; The Rhizobiaceae. In: Bergey's Manual of Determinative Bacteriology, 8th edition. (R. E. Buchanan and N. E. Gibbons, eds) pp 261-267. Williams and Wilkins, Baltimore.

Josey, D. P., J. L. Beynon, A. W. B. Johnston and J. E. Beringer, (1979): Strain identification in *Rhizobium* using intrinsic antibiotic resistance. Journal of Applied Bacteriology, 46: 343-350.

Karargatzides, J. D., M. C. Lewis and H. M. Schulman, (1985): Nitrogen fixation in the high arctic at Sarcpa Lake, Northwest Territories. Canadian Journal of Botany, 63: 974-979.

Karnovsky, M. J., (1965): A formaldehyde-glutaraldehyde fixative of high for use in electron microscopy. Journal of Cell Biology, 27: 137a-138a.

Kharbush, S. S., (1928): Reserches sur les tubercules radicaux de qu'ilques Papilionacees alpines. Bull. Soc. Bot. Fr., 75: 674-696.

Lamourex, G. and M. M. Grandtner, (1977): Contribution a l'etude ecologique des dunes mobiles. Les elements

phytosociologiques. Canadian Journal of Botany, 55: 158-171.

Lawrence, J. V. and S. Maier, (1977): Correction for the inherent error in optical density readings. Applied and Environmental Microbiology, 33: 482-484.

Libbenga, K. R. and R. J. Bogers, (1974): Root nodule morphogenesis. In: The Biology of Nitrogen Fixation, (A. Quispel, ed.), 430-472. North-Holland Publ. Co., Amsterdam.

Lim, G. and J. C. Burton, (1982): Nodulation status of the Leguminosae. In: Nitrogen Fixation Volume 2: *Rhizobium*, (W. J. Broughton, ed.) pp 1-34, Clarendon Press, Oxford.

Lindstrom, K. and S. Lehtomaki, (1988): Metabolic properties, maximum growth temperature and phage sensitivity of *Rhizobium* sp. (Galega) compared with other fast-growing rhizobia. FEMS Microbiology Letters 50: 277-287.

Little, M. J. and E. J. Hills, (1978): Agricultural Experimentation: Design and Analysis. John Wiley and Sons, Toronto.

Mani, M. S., (1980): Introduction. In: Ecology of Highlands. Monographicae Biologicae, Vol 40, (J. Illies, ed), 1-9 pp. Dr W Junk bv, The Hague.

Minchin, F. R., J. E. Sheehy, and J. F. Witty, (1986): Further errors in the acetylene reduction assay: effects of plant disturbance. *Journal of Experimental Botany* 37 (183): 1581-1591.

Mortensen, L. E., (1961): A simple method for measuring nitrogen fixation by cell-free enzyme preparations of *Clostridium pasteurianum*. *Analytical Biochemistry*, 2:216-220.

Mortenson, L. E., (1964): Ferredoxin and ATP, requirements for nitrogen fixation in cell-free extracts of *Clostridium pasteurianum*. *Proceedings of the National Academy of Science, U.S.*, 52: 272-279.

Munns, D. N., (1977): In Exploiting the legume-*Rhizobium* in tropical agriculture, (Vincent, M. J., A. S. Whitney and J. Bose, eds.), pp 211-238. College of Tropical Agriculture, Misc. Publ. # 145, University of Hawaii, Honolulu.

Newcomb, W., (1976): A correlated light and electron microscopic study of symbiotic growth and differentiation in *Pisum sativum*. *Canadian Journal of Botany*, 26: 60-69.

Newcomb, W., (1981): Nodule morphogenesis and differentiation. *International Review of Cytology*, 13: 1-14.

Newcomb, W. and R. L. Peterson, (1979): The occurrence and ontogeny of transfer cells associated with lateral roots and root nodules in Leguminosae. Canadian Journal of Botany, 57 (23): 2583-2602.

Newcomb, E. H. and S. R. Tandon, (1981): Uninfected cells of soybean root nodules: ultrastructure suggests key role in ureide production. Science, 212: 1394-1396.

Newcomb, W. and S. M. Wood, (1986): Fine structure of nitrogen-fixing leguminous root nodules from the Canadian Arctic. Nordic Journal of Botany, 6: 609-626.

Nicholas, D. J. D., D. J. Silvester and J. F. Fowler (1961): Use of radioactive nitrogen in studying nitrogen fixation in bacteria cells and their extracts. Nature, 189: 634-636.

Niles, A. M. (1983): Some Aspects of Symbiotic Nitrogen Fixation in *Vicia cracca* L. (Leguminosae). M.Sc. Thesis, Memorial University of Newfoundland, St. John's, Newfoundland.

Norris, D. O., (1965): Acid production by *Rhizobium*, a unifying concept. Plant and Soil, 77: 29-38.

Okafor, N. and M. Alexander, (1975): Preliminary physiological studies on cowpea rhizobia. Soil Biology and Biochemistry, 7:

405-406.

Padmanabhan, S., R. D. Hirtz and W. J. Broughton, (1990): Rhizobia in tropical legumes: cultural characteristics of *Bradyrhizobium* and *Rhizobium* sp. Soil Biology and Biochemistry, 22 (1): 22-28.

Pankhurst, C. E. (1977): Symbiotic effectiveness of antibiotic resistant mutants of fast and slow growing strains of *Rhizobium* nodulating *Lotus* species. Canadian Journal of Microbiology, 23: 1026-1033.

Parker, C. A. and P. H. Graham, (1964): Diagnostic features in the characterisation of the root nodule bacteria of legumes Plant and Soil 20: 383-396.

Pate, J. S. and B. E. S. Gunning, (1972): Transfer cells. In: Annual Review of Plant Physiology, (L. Machlis, ed.), 23: 173-196. Annual Reviews Inc., Palo Alto, CA.

Pate, J. S., B. E. S. Gunning and L. G. Briarty, (1969): Ultrastructure and functioning of the transport system of the leguminous root nodule. Planta (Berl.), 85: 11-34.

Polunin, N., (1959): Circumpolar Arctic Flora. Oxford Clarendon Press, London.

Postgate, J. R. (1980): Foreword. In: Recent Advances in Biological Nitrogen Fixation, (N. S. Subba Rao, ed.), pp v-xiv. Edward Arnold Ltd., London.

Prévost, D. and A. K. Bal, (1989): Morphological development and lipid composition of nodules from the arctic legume *Oxytropis arctobia*. Proceedings of the North American Symbiotic Nitrogen Fixation Conference, pp 112, Ames, Iowa.

Prévost, D., L. M. Bordeleau, S. Caudry-Reznick, H. M. Schulman and H. Antoun, (1987): Characteristics of rhizobia isolated from three legumes indigenous to the Canadian high arctic: *Astragalus alpinus*, *Oxytropis maydelliana* and *Oxytropis arctobia*. Plant and Soil, 98: 313-324.

Pugashetti, B. K., J. S. Angle and G. H. Wagner, (1982): Soil microorganisms antagonistic towards *Rhizobium japonicum*. Soil Biology and Biochemistry, 14: 45-49.

Robertson, J. G. and P. Lyttleton, (1982): Coated and smooth vesicles in the biogenesis of cell walls, plasma membranes, infection threads and peribacteroid membranes in root hairs and nodules of white clover. Journal of Cell Science, 58: 63-78.

Saito, S. M. T., E. Matsui and E. Salati, (1980): $^{15}\text{N}_2$

fixation, H_2 evolution and C_2H_2 reduction relationships in *Phaseolus vulgaris*. *Physiologia Plantarum*, 49: 37-42.

Schollhorn, R. and R. H. Burrris, (1967): Acetylene as a competitive inhibitor of nitrogen fixation. *Proc. Natl. Acad. Sci. U.S.*, 58: 213-216.

Schulman, H. M., M. C. Lewis, E. M. Tipping and L. M. Bordeleau, (1988): Nitrogen fixation by three species of leguminosae in the Canadian high arctic tundra. *Plant, Cell and Environment*, 11: 721-728.

Scoggan, H. J., (1950): The Flora of Canada, Part 3 Dicotyledoneae, National Museum of Natural Sciences, Publications in Botany 7 (3), Ottawa.

Siddique, A. M. and A. K. Bal, (1991): Nitrogen fixation in peanut and detopped conditions with special reference to lipid bodies. *Plant Physiology*, 95: 896-899.

Smibert, R. M. and N. R. Krieg, (1981): General characterization. In: *Manual of Methods for General Bacteriology*. (P. Gerhardt, ed.), pp 409-443. American Society of Microbiology.

Somerville, C. and J. Browse, (1991): Plant lipids: metabolism, mutants and membranes. *Science*, 252: 80-87.

Sprent, J. I., (1972): The effects of water stress on nitrogen-fixing root nodules II. Effects on the fine structure of detached soybean nodules. *New Phytol*, 71: 443-450.

Sprent, J. I., (1979): The Biology of Nitrogen-fixing Organisms. McGraw-Hill, London.

Sprent, J. I., (1981): Adaptive variation in legume nodule physiology resulting from host rhizobial interactions. In: Nitrogen As an Ecological Factor. (J. A. Lee, S. Mcneil and I. H. Rorison, eds). Blackwell Scientific Publications, Oxford.

Sprent, J. I., (1989): Which steps are essential for the formation of functional legume nodules? *New Phytology*, 111: 129-153.

Spurr, A. R., (1969): A low viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research*, 26: 31-43.

Steinborn, J. and R. J. Roughley, (1974): Sodium chloride as a cause of low numbers of *Rhizobium* in legume inoculants. *Journal of Applied Bacteriology* 37: 93-99

Stowers, M. D. and R. J. A. Eaglesham, (1984): Physiological and symbiotic characterization of fast-growing *Rhizobium japonicum*. Plant and Soil, 77, 3-14.

Subba Rao, N. S., (1980): Chemically and biologically fixed nitrogen: potential and prospects. In: Recent Advances in Biological Nitrogen Fixation, (N. S. Subba Rao, ed.), 1-7 pp. Edward Arnold Ltd., London.

Subba Rao, N. S., (1988). Biological nitrogen fixation: potentialities, prospects and limitations. In: Biological Nitrogen Fixation, Recent Developments (N. S. Subba Rao, ed.) pp 1-19. Gordon and Breach, New York.

Trinick, M. J., (1982): Biology. In: Nitrogen Fixation Volume 2: *Rhizobium* (W. J. Broughton, ed.) pp 76-146, Clarendon Press, Oxford.

Trinick, J., M. J. Dilworth and M. Grounds, (1976): Factors affecting the reduction of acetylene by root nodules of *Lupinus* species. New Phytologist, 77: 359-370.

Trunchet, G., (1978): Sur l'état déployé des meristème des nodules radiculaires des légumineuses. Annales des Sciences Naturelles Botanique, Paris, 19: 3-38.

Turner, G. L. and A. H. Gibson, (1980): Measurement of nitrogen fixation by indirect means. In: Methods for Evaluating Biological Nitrogen Fixation. (F. J. Bergersen, ed.) pp 111-128. John Wiley and Sons, Toronto.

Vincent, J. M., (1970): A Manual for the Practical Study of the Root Nodule Bacteria. Blackwell, Oxford.

Vincent, J. M., (1974): Root-nodule symbioses with *Rhizobium*. In: The Biology of Nitrogen Fixation, (A. Quipel, ed.), 263-341 pp. North-Holland Publ. Co., Amsterdam.

Vincent, J. M., (1977): *Rhizobium*: General Microbiology. In: A Treatise on Dinitrogen Fixation, Section III Biology. (R. W. F. Hardy and W. S. Silver, eds.), pp 277-366. John Wiley and Sons, Toronto.

Werner, D., (1987): Pflanzliche und mikrobielle symbiosen. G. Thieme Verlag, Stuttgart.

Wilson, J. K., (1939): Leguminous plants and their associated organisms. Cornell University Agricultural Experimental Station Memoirs # 221, pp 48.

Yates, M. J., (1980): Biochemistry of nitrogen fixation. In: The Biochemistry of Plants (B. J. Miflin), pp 1-64. Academic

Press, New York.

